# Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/003567

International filing date: 04 February 2005 (04.02.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/541,999
Filing date: 04 February 2004 (04.02.2004)

Date of receipt at the International Bureau: 11 March 2005 (11.03.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

1291338

# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

*March 02, 2005*

**THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.**

**APPLICATION NUMBER:** *60/541,999*
**FILING DATE:** *February 04, 2004*
**RELATED PCT APPLICATION NUMBER:** *PCT/US05/03567*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

Please type a plus sign (+) inside this box → [+]

PTO/SB/16 (5-03)
Approved for use through 04/30/2003. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE



# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

**This is a request f r filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).**



## INVENTOR(S)

| Given Name (first and middle [if any]) | Family Name or Surname | Residence (City and either State or Foreign Country) |
|---|---|---|
| Ryan Smith | Westberry | Westminster, CO U.S.A. |

☒ *Additional inventors are being named on th* 1 *separately numbered sheets attached hereto*

**TITLE OF THE INVENTION (280 characters max)**

METHODS AND COMPOSITIONS TO ENHANCE AMPLIFICATION EFFICIENCY AND SIGNAL

*Direct all correspondence to:* **CORRESPONDENCE ADDRESS**

☒ Customer Number 20686 →

*OR* *Type Customer Number here*

*Place Customer Number Bar Code Label here*

| | | | | | |
|---|---|---|---|---|---|
| ☐ Firm *or* Individual Name | | | | | |
| Address | | | | | |
| Address | | | | | |
| City | | State | | ZIP | |
| Country | | Telephone | | Fax | |

**ENCLOSED APPLICATION PARTS *(check all that apply)***

| | | | |
|---|---|---|---|
| ☒ Specification *Number of Pages* | 41 | ☐ CD(s), Number | |
| ☒ Drawing(s) *Number of Sheets* | 26 | ☐ Other (specify) | |
| ☒ Application Data Sheet. See 37 CFR 1.76 | | | |

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

| | | FILING FEE AMOUNT ($) |
|---|---|---|
| ☐ | A check or money order is enclosed to cover the filing fees | |
| ☒ | The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number 04-1415 | $160.00 |
| ☐ | Payment by credit card. Form PTO-2038 is attached. | |

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

*Respectfully submitted,*

SIGNATURE

TYPED or PRINTED NAME Paul J. Pr ndergast

TELEPHONE 303-629-3400

Date 02/04/04

REGISTRATION NO. *(if appropriate)* 46,068

Docket Number: 34092/US

## USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

| CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10)<br>Applicant(s): **Ryan Smith WESTBERRY et al.** | | | Docket No.<br>**34092/US** |
|---|---|---|---|
| Serial No.<br>**not yet assigned** | Filing Date<br>**not yet assigned** | Examiner<br>**not yet assigned** | Group Art Unit<br>**not yet assigned** |

Invention: **METHODS AND COMPOSITIONS TO ENHANCE AMPLIFICATION EFFICIENCY AND SIGNAL**

I hereby certify that the following correspondence:

**Provisional Application for Patent Cover Sheet; Provisional Application (Specification, Claims & Abstract 41 pages); 26 Sheets of Drawings; Application Data Sheet; Certificate of Mailing by Express Mail; Return Acknowledgement Postcard**

*(Identify type of correspondence)*

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on

**February 4, 2004**

*(Date)*

**Paul J. Prendergast/Dorsey & Whitney LLP**

*(Typed or Printed Name of Person Mailing Correspondence)*

*(Signature of Person Mailing Correspondence)*

**EV 016 647 852 US**

*("Express Mail" Mailing Label Number)*

**Note: Each paper must have its own certificate of mailing.**

# METHODS AND COMPOSITIONS TO ENHANCE AMPLIFICATION EFFICIENCY AND SIGNAL

## FIELD OF THE INVENTION

The invention generally relates to methods and compositions for increasing amplification efficiency in nucleic acid amplification reactions, for increasing the stability of amplification reaction mixtures over repeated freeze/thaw cycles, and/or for improving the signal to noise ratio of amplification detection methods such as, e.g., real time PCR.

## BACKGROUND OF THE INVENTION

The ability to prepare large amounts of nucleic acid molecules is requisite to a number of protocols in molecular biology, as well as a basic requirement in numerous downstream uses in biotechnology and clinical research. For example, amplified nucleic acid molecules are often used in cloning experiments, DNA sequencing reactions, restriction digestion reactions, and subsequent ligation reactions, and these uses are all, or to some extent, dependent on quality and quantity of the starting DNA material. As such, there has, and continues to be, a need for reliable methods for preparing large amounts of quality, specific nucleic acid molecules.

In addition, the ability to detect and quantify nucleic acid molecules from a starting material is useful in a number of clinical, industrial and basic research application. For example, sensitive and accurate detection and quantification of viral nucleic acid sequences in a starting sample is helpful in a clinical setting for accurate diagnosis and subsequent treatment of the patient. As such, there has, and continues to be, a need for facilitating detection and quantification of a nucleic acid sequence from a starting material.

The predominant approach for amplifying nucleic acid is via the polymerase chain reaction (PCR). PCR is a convenient *in vitro* amplification method useful in the exponential increase of template nucleic acid. Particular applications of PCR are useful in the detection/quantification of target gene expression and confirmation of differential expression of target genes detected using array techniques (generally termed real time-PCR or kinetic PCR). In general, optimization of PCR techniques could facilitate both the accuracy and total levels or amounts of the amplified product (amplicon), and optimization of real time-PCR techniques could facilitate the sensitivity

and suppress nonspecific amplification during the procedure (note that nonspecific amplification is particularly problematic when the starting material is small). In addition, there is a need for greater stability in PCR reaction conditions both during the reaction and during storage of the relevant constituents of the reaction.

Conventionally PCR optimization has focused on modifying standard PCR buffers, altering primer annealing temperatures, providing more effective thermostable polymerase enzymes, and designing more effective primer molecules. With regard to product quantification in real-time PCR, optimization has focused on the development of two relatively new assays: the TaqMan method of real time PCR (Holland et al. 1991) and Binary hybridization probes (Lee et al., 1993). Each real time assay relies upon the release of a detectable signal upon production of a PCR product. In either situation, reduction of non-specific amplification and facilitation of the enzyme stability useful in both PCR and real time-PCR is critical.

As such, there is a continuing need in the art for improvement of PCR techniques and compositions that allow for more sensitive, accurage and robust results.

Against this backdrop the present invention has been developed.

## SUMMARY OF THE INVENTION

The present invention provides compositions and methods for improving the outcome of nucleic acid amplification reactions typically comprising at least one cycle of a denaturing step, an annealing step, and an extension step, through the addition of at least one of a polyol, an anti-freeze protein or a dUTP/dTTP mixture to the reaction buffer prior to or contemporaneous with the initial denaturing step.

In one aspect, methods and compositions for increasing amplicon yield are provided, comprising the addition of at least one of a polyol and/or an anti-freeze protein to the reaction mixture. In one embodiment, addition of the polyol lowers the denaturation temperature of the template nucleic acid. In a preferred embodiment, the polyol is included in a reaction mixture comprising

a template nucleic acid having a degree of secondary structure. In a particularly preferred embodiment, the polyol is selected from the group consisting of sorbitol and mannitol. Ancillary materials can be included with the polyols in PCR, including, but not limited to, DMSO, glycerol, single-stranded binding protein, trehalose, n-propyl sulfoxide, and the like, to further facilitate amplification by the polyols.

In another embodiment, addition of the anti-freeze protein improves the signal-to-noise ratio of the reaction. Preferably, the anti-freeze proteins have one or more alanine-rich motifs for enhancement of amplification signal and sensitivity. In particularly preferred embodiments, a carrier protein, e.g., BSA, is included with the anti-freeze protein in the reaction mixture to maximize the effect.

In another aspect, methods and compositions for inhibiting primer-dimer formation in a nucleic acid amplification reaction are provided, comprising the addition of a dUTP/dTTP combination to the reaction mixture. In one embodiment, a percentage of dTTP in a standard dNTP mix is replaced with dUTP or other like dNTP analogs, and the modified dNTP mixture is then used in the amplification reaction. Compositions in accordance with this embodiment include dNTP mixtures having up to 50% of the dTTP replaced by dUTP.

The present invention also provides methods and compositions for improving the stability of the reaction mixture in a nucleic acid amplification reaction which includes at least one freeze-thaw cycle, through the addition of at least one of a polyol or an anti-freeze protein to the reaction buffer prior to or contemporaneous with the freeze-thaw cycle.

The present invention further provides improved nucleic acid amplification reaction mixtures and buffers for use in nucleic acid amplification reactions, comprising at least one of a polyol, an anti-freeze protein and a dUTP/dTTP mixture. In a preferred embodiment, one or more of these enhancers are added to a reaction mixture comprising a zwitterionic buffer. As detailed herein, these novel amplification reaction mixture components may improve amplicon yield as well as signal intensity in quantification reactions, enhance sensitivity of the amplification reaction, and stabilize the reaction mixture during freeze-thaw events.

Exemplary embodiments of the present invention include compositions for maximizing signal amplification during real time PCR where release of fluorescent signal upon 5'-3' nucleolytic degradation is anticipated. Preferred embodiments include anti-freeze proteins, typically having one or more AFP type I alanine motifs, in combination with a zwitterionic buffer formulation with a pH between about 7.9 and about 8.1. In a preferred embodiment, the nucleic acid amplification reaction is real time PCR and the addition of the anti-freeze protein enhances signal amplification These and various other features and advantages of the invention will be apparent from a reading of the following detailed description and a review of the appended claims.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a stained 1% agarose gel showing the reaction products from PCR where varying percentages of dTTP in the dNTPs was replaced with dUTP. The incorporation of dUTP into the reaction mixes significantly reduces the amount of primer-dimer formed during each reaction.

Figure 2 is a stained 1% agarose gel showing the reaction products from PCR comparing a control reaction to a reaction where 20% of the dTTP was replaced by dUTP. Replacement of dTTP with dUTP significantly reduced the amount of primer-dimer formed during PCR.

Figure 3 graphically represents melt curve comparisons for dNTP analogs and mixtures.

Figures 4A and B graphically illustrates that replacement of dTTP with dUTP does not significantly affect Ct or RFU during real time PCR.

Figures 5A and B graphically show the Beta-Actin sequence used for the sorbitol secondary structure assays, Figure 5A shows the secondary structure of the gene at 37, 55 and 68°C in the absence of a extremely GC rich region, and Figure 5B shows the same gene with the included GC rich region that promotes secondary structure even at 37, 55 and 68°C.

Figure 6 is a stained 1% agarose gel showing the reaction products from PCR on the templates of Figure 5A and 5B in the presence of increasing amounts of sorbitol. The upper band is amplified

product from the secondary structure containing molecule, indicating melting of the secondary structure, and the lower band is amplified product from the non-secondary structure containing molecule, indicating a control level of amplification of the non-secondary structure containing template.

Figures 7A, 7B and 7C are as above in Figure 6, except PCR was performed in a combination of several chemical agents, including sorbitol, mannitol, dUTP, DMSO, single-stranded binding protein and trehalose.

Figures 8A and 8B graphically represent that inclusion of 100mM sorbitol in RT-PCR Master Mix facilitates storage stability at -20°C. Figure 8A shows Ct values and Figure 8B shows RFU values from PCR performed after freeze-thaw on sorbitol containing and non-containing master mix buffers for one or three weeks. Samples were either stored at 4, -20 or -80°C.

Figures 9A and 9B are stained 1% agarose gels showing RT PCR reaction products after one-week (9A) or three-week (9B) of storage at either 4, -20 or -80°C in zero, 100mM, or 200mM sorbitol.

Figure 10 is a stained 1% agarose gel showing yields of RT-PCR reaction products from reactions having from 0 to 200 μg/ml anti-freeze protein 1 included within the reaction.

Figures 11 A and B graphically illustrate signal amplification synergy between anti-freeze protein 1 and BSA during RT-PCR (A) Average RFU and (B) Threshold Cycle (Ct).

Figures 12A and 12B graphically illustrate that inclusion of BSA and AFP1 in RT-PCR Master Mix facilitates storage of the buffer stability at -20°C. Figure 12A shows average RFU values and Figure 12B shows threshold cycle values. Buffer samples were either BSA alone, AFP-1 alone, BSA and AFP-1 or no additives.

Figures 13A and 13B graphically illustrate PCR buffer compositions including AFP1, BSA, and assorted combinations of buffer and salt, (A) threshold cycle and (B) average RFU.

Figure 14 graphically compares the threshold cycle for Real-Time PCR products prepared in TAPS-KOH, pH8, TAPS-tris, pH8 and RealMaster 10X standard pH 8.4.

Figure 15 graphically compares the threshold cycle for Real-Time PCR products prepared in RealMaster 10X standard buffer at pH 8.4, TAPS-tris KCL buffer at pH 8, and Invitrogen Platinum qPCR Supermix-UDG, used according to manufacturers standards.

Figure 16 graphically compares the threshold for real-time PCR products prepared in the presence of no additives, BSA, AFGP, AFGP + BSA, AFP1 + BSA, AFGP + AFP1 + BSA.

Figure 17 graphically compares RFU for long term stability testing of an embodiment of the present invention as compared to testing performed with the Invitrogen Platinum qPCR Supermix UDG product, purchased and performed using Invitrogen protocol.

**DETAILED DESCRIPTION OF THE INVENTION**

The following definitions are provided to facilitate understanding of certain terms used frequently herein and are not meant to limit the scope of the present disclosure:

As used herein, "antisense" refers to polynucleotide sequences that are complementary to target "sense" polynucleotide sequences.

As used herein, "Ct shift" or "threshold cycle" refers to the cycle at which an amplification product is detectable, a Ct shift of 1.5 to 3 cycles is equivalent to an approximate 5 to 10 fold higher input amount of template DNA.

As used herein, "extrachromosomal DNA" refers to certain integrated and non-integrated episomal genetic elements, e.g., certain DNA viruses as well as natural plasmids, plasmids derived cloning and expression vectors, natural bacterial phages and cloning and expression phages, yeast artificial chromosomes (YAK), bacterial artificial chromosomes (BAC), two micron DNA, two-micron derived yeast shuttle vectors, mitochondrial DNA, cosmids, phagemids, transposons, and the like.

As used herein, "nucleic acid" or "NA" refers to both a deoxyribonucleic acid (DNA) and a ribonucleic acid (RNA), as well as modified and/or functionalized versions thereof. Similarly, the term "nucleotide" as used herein includes both individual units of ribonucleic acid and deoxyribonucleic acid as well as nucleoside and nucleotide analogs, and modified nucleotides such as labeled nucleotides. In addition, "nucleotide" includes non-naturally occurring analog structures, such as those in which the sugar, phosphate, and/or base units are absent or replaced by other chemical structures. Thus, the term "nucleotide" encompasses individual peptide nucleic acid (PNA) (Nielsen et al., Bioconjug. Chem. 1994; 5(1):3-7) and locked nucleic acid (LNA) (Braasch and Corey, Chem. Biol. 2001; 8(1):1-7) units.

As used herein, "polynucleotide," "oligonucleotide" or grammatical equivalents thereof means at least two nucleotides covalently linked together. As will be appreciated by those of skill in the art, various modifications of the sugar-phosphate backbone may be done to increase the stability of such molecules in physiological environments, including chemical modification such as, *e.g.*, phosphorothioate or methyl phosphonate. Further, such molecules may be functionalized by coupling with one or more molecules having distinct characteristic properties for purposes of, *e.g.*, facilitating the addition of labels.

As used herein, "nucleic acid sequence" refers to the order or sequence of nucleotides along a strand of nucleic acids. In some cases, the order of these nucleotides may determine the order of the amino acids along a corresponding polypeptide chain. The nucleotide sequence thus codes for the amino acid sequence. The nucleic acid sequence may be single-stranded or double-stranded, as specified, or contain portions of both double-stranded and single-stranded sequences. The nucleic acid sequence may be composed of DNA, both genomic and cDNA, RNA, or a hybrid, where the sequence comprises any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil (U), adenine (A), thymine (T), cytosine (C), guanine (G), inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc.

As used herein, "complementary" or "complementarity" refers to the ability of a nucleotide in a polynucleotide molecule to form a base pair with another nucleotide in a second polynucleotide molecule. For example, the sequence 5'-A-C-T-3' is complementary to the sequence 3'-T-G-A-5'. Complementarity may be partial, in which only some of the nucleotides match according to

base pairing, or complete, where all the nucleotides match according to base pairing. For purposes of the present invention "substantially complementary" refers to 95% or better complementarity over the length of the target base pair region.

As used herein, "expression" refers to transcription and translation occurring within a host cell. The level of expression of a DNA molecule in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present within the cell or the amount of DNA molecule encoded protein produced by the host cells. Further detail for the term "expression" within the context of the present invention can be obtained via a review of Sambrook et al., 1989, Molecular Cloning; A Laboratory Manual; 18.1-18.88.

As used herein, "freeze/thaw" conditions are characterized by freezing a target material at a temperature typically below 0°C and preferably at a temperature not to go below about -30°C to about -40°C. Thaw conditions typically do not exceed room temperature. A typical slow-freeze/thaw cycle is where a material is frozen between about 0°C and about -40°C, stored at that temperature for some period of time, and thawed between about 1°C to about 27°C, dependent on the end use required.

As used herein, "host cell" or "host cells" refers to cells expressing or capable of expressing a heterologous polynucleotide molecule, for example a plasmid vector. Host cells of the present invention express polynucleotides encoding polypeptides useful in any number of uses, including biotechnological, molecular biological and clinical settings. Examples of suitable host cells in the present invention include, but are not limited to, bacterial, yeast, insect and mammalian cells. Specific examples of such cells include, *E. Coli* DH5α cells, as well as various other bacterial cell sources, for example the *E. Coli* strains: DH10b cells, XL1Blue cells, XL2Blue cells, Top10 cells, HB101 cells, and DH12S cells, and yeast host cells from the genera including *Saccharomyces*, *Pichia*, and *Kluveromyces*.

As used herein, "isolated" and "purified" for purposes of the present invention are interchangeable, and refer to a polynucleotide, for example extrachromosomal nucleic acid, that has been separated from cellular debris, for example, high molecular weight DNA, RNA and protein. This would include an isolated RNA sample that would be separated from cellular debris, including DNA.

As used herein, "protein," "peptide," and "polypeptide" are used interchangeably to denote an amino acid polymer or a set of two or more interacting or bound amino acid polymers.

As used herein, "real-time PCR" refers to quantitative PCR techniques that typically use fluorescence probes, beacons, and/or intercalating dyes during all cycles of the process.

As used herein, "selectable marker" refers to a gene on extrachromosomal DNA typically used to select a target extrachromosomal DNA. Selectable markers include, for example, genes that encode antimetabolite resistance such as the DHFR protein that confers resistance to methotrexate (Wigler et al., 1980, Proc Natl Acad Sci USA, 77:3567; O'Hare et al., 1981, Proc Natl Acad Sci USA, 78:1527), the GPT protein that confers resistance to mycophenolic acid (Mulligan & Berg, 1981, PNAS USA, 78:2072), antibiotic resistance, *i.e.*, neomycin resistance marker that confers resistance to the aminoglycoside G-418 (Calberre-Garapin et al., 1981, J Mol Biol, 150:1).

As used herein, "stringency" refers to the conditions, *i.e.*, temperature, ionic strength, solvents, and the like, under which hybridization between polynucleotides occurs. Hybridization being the process that occurs between the primer and template DNA during the annealing step of the amplification process.

As used herein, "vector," "extra-chromosomal vector" or "expression vector" refers to a circular polynucleotide molecule, usually double-stranded, which may have a site for insertion or have inserted a target heterologous polynucleotide. The heterologous polynucleotide molecule may or may not be naturally found in the host cell, and may be, for example, one or more additional copy of the heterologous polynucleotide naturally present in the host genome. The vector is adapted for transporting the foreign polynucleotide molecule into a suitable host cell. Once in the host cell, the vector may be capable of integrating into the host chromosomes. The vector may optionally contain additional elements for selecting cells containing the integrated polynucleotide molecule as well as elements to promote transcription of mRNA from transfected DNA. Examples of vectors useful in the methods of the present invention, include, but are not limited to, plasmids, bacteriophages, cosmids, retroviruses, and artificial chromosomes.

As used herein, "weak organic acid" refers to and includes but is not limited to, bicine, tricine, TAPSO, CAPSO, EPPS, Hepes, CHES, Taurin, MOPS, AMPSO. In general, weak organic acids have a pK between about 8 and 9.

As used herein, "weak organic base" generally refers to and includes but is not limited to, tris, bis-tris, imidazole, and bis-tris-propane. In general weak organic bases have a pK between 8 and 9 for purposes of the present invention, although slight modifications above 8 and 9 are envisioned to be within the scope to the disclosure.

Embodiments of the present invention provide methods and compositions for enhancing the overall efficiency of an amplification reaction, and preferably provide methods and compositions for enhancing the outcome and overall efficiency of nucleic acid amplification reactions such as, *e.g.,* standard PCR, real-time PCR or both standard and real-time PCR. Embodiments are directed toward facilitating the specificity of PCR, at enhancing the sensitivity of PCR, at enhancing the signal amplification of PCR, and/or at providing enhanced storage stability for amplification buffers, e.g., PCR buffers, that undergo numerous freeze-thaw cycles. Embodiments of the present invention include the following:

replacement of a portion of dTTP in a standard dNTP mix useful in amplification reactions with dUTP to reduce primer-dimer formation;

inclusion of sorbitol or other like polyol material in a PCR buffer to both enhance the product yield and increase the stability of the buffer during freeze thaw reactions;

inclusion of anti-freeze proteins (AFP) to enhance both signal amplification (RFU) and sensitivity (threshold cycle) during real-time PCR;

inclusion of AFP within amplification buffers to enhance storage stability of the buffer during freeze/thaw conditions;

preferable inclusion of a carrier protein, for example BSA, with the AFP to provide an additional effect on signal size and sensitivity, as well as on storage stability; and

inclusion of dUTP, sorbitol, and AFP in a zwitterionic buffer formulation, *i.e.*, TAPs-tris KCL or TAPS-KOH KCL based buffer to provide a high performance PCR buffer (increases sensitivity, specificity, signal size and storage stability), especially where the pH is between 7.9 and 8.1.

**Primer Incorporation of dUTP Or Other Analogs To Reduce Primer-Dimer Formation**

The present invention provides modified dNTP mixes and corresponding methods using dNTP containing mixes, for limiting or reducing the formation of primer-dimers during nucleic acid amplification reactions, *e.g.*, PCR, real-time PCR, etc. In one embodiment, a portion of the dNTP mixture in the reaction mixture is replaced with an appropriate dNTP analog, *e.g.*, replace dTTP with dUTP. Standard concentrations of dNTPs are used as a starting point within the target reactions as is well known in the art, i.e., approximately 200μM per dNTP for PCR. In one embodiment, from 10% to 50% of the dTTP in a standard dNTP mix is replaced with dUTP, and preferably from about 20% to about 40% of the dTTP in the standard dNTP mix is replaced with dUTP. Other embodiments of the present invention include the use of other dNTP analogs within the dNTP mix, for example dITP, deaza-dGTP, and mixtures thereof.

Note that the presence of dNTP analogs, e.g., dUTP, promote the fill-in reaction to be completed with a minimum number of primer-dimers formed. In general, inclusion of a dNTP analog is less thermodynamically favorable for incorporation based on annealing temperatures used in standard PCR. Where a fill-in reaction is not required, the benefit of inclusion of a dNTP analog is limited. As such, primer fill-in reactions result in primer-dimers are reduced in number. Note that prior art usage of dUTP focused on incorporation of dUTP into product (amplicon) and subsequent degradation of such product with UNG. UNG treatment was performed on PCR reactions before dUTP was added to ensure that any contaminate UTP containing product was degraded before the next reaction, thereby enhancing the reaction specificity.

In an alternative embodiment, primers are designed to exhibit dNTP analogs in place of standard dNTPs in order to reduce the thermal-dynamic favorability of primer-dimer formation. For example, dNTP analogs, *e.g.*, dUTP, in the context of primer-dimer formation, will tend to form weaker interactions as compared to dTTP at the same positions. This reduction in interaction strength is relevant when the level of mispairing is high or the number of base-pair overlap is small, *i.e.*, conditions that favor primer-dimer formation. The same dNTP analog containing primers have sufficient interaction strength with their target template site to remain hybridized under the proper temperature cycling PCR conditions. As such, primer design that includes

dNTP analog incorporation selectively favors a reduction in the formation of primer-dimers, while maintaining the relative hybridization strength of the primer with its template target site. In one embodiment of the invention, all the dTTP in the primer is replaced with dUTPs.

**Polyols Facilitate The PCR of Template NA Having Secondary Structure**

Polyols, *i.e.*, alcohol derivatives of monosaccharide, facilitate amplification of template nucleic acid molecules that include some level of secondary structure. In one embodiment of the invention, one or more polyol compounds is included within a standard amplification reaction to enhance the sensitivity of amplification on target template NA where the NA has some degree of secondary structure. Without being bound by theory, it is believed that the polyol acts as a chemical melting agent on the template nucleic acid, thereby facilitating amplification of the template at lower denaturation temperatures.

The present invention preferably includes one or more polyols in a reactionbuffer or mixture. In particular, the present invention provides for the inclusion of polyols in amplification reactions where the target amplification sequence has increased levels of secondary structure. Illustrative polyols for use in the present invention include, but are not limited to, glycerol, sorbitol, mannitol, PEG, etc. In preferred embodiments the polyol is sorbitol or mannitol, alone or in combination. Polyol concentration in amplification buffers can range from about 100mM to about 500mM, preferably is from about 50mM to about 400mM, and is most preferably from about 100mM to about 300mM. Inclusion of other melting or disruptive agents in combination with the polyol during amplification is anticipated, for example, inclusion of from 1% to 5% DMSO, from 50ng to about 500ng single-stranded binding protein, from about 1% to about 5% n-propyl sulfoxide solution, from about 100mM to 500mM trehalose, and up to 25% replacement of the dTTP with dUTP in the dNTP mix, are all within the scope of the present invention.

In another embodiment of the present invention, inclusion of one or more polyols in a nucleic acid amplification buffer provides increased stability of the buffer under freeze/thaw conditions. In a preferred embodiment the amplification buffer is a standard PCR buffer. In one embodiment, sorbitol or other like polyol is included in a PCR buffer at a concentration of from about 50mM to about 500mM, preferably is from about 50mM to about 400mM, and is most preferably from about 100mM to about 300mM. As above, inclusion of other disruptive agents,

i.e., DMSO, SSBP, and the like, with th polyol is anticipated to enhance the overall positive affect.

Without being bound by theory, it is believed that the inclusion of a polyol in the reaction mixture serves at least the two-fold effect of increasing the yield of amplicon during amplification of template molecules having some level of secondary structure and of additionally maintaining the stability of the buffer during multiple cycles of freeze/thaw.

Any reasonable source of polyol can be used in the present invention, for example sorbitol can be obtained from Sigma/Fluka and mannitol can be obtained from Sigma/Fluka.

**Anti-Freeze Proteins (AFP)**

Anti-freeze proteins (AFPs) represent a family of proteins that contribute freeze resistance and freeze tolerance to a number of species that thrive in freezing conditions. In general, AFP molecules within the organism bind to the surface of seed ice crystals and control the crystal's growth. *See* Jia et al., (2002) TRENDS in Biochem. Sciences, 27(2) 101-106. A number of different organisms have been found to express AFPs, including several species of fish and several species of insect. Fish AFPs are classified into five groups (see Table 1), based on predominately primary and secondary structure analysis studies. With regard to insect AFPs, several insect species, including Tm or Dc (beetle) and Cf (moth), have been classified into two groups (note that AFPs have been isolated from several plant species as well).

Importantly, several different structural motifs between fish group members function similarly to inhibit ice growth, including an alanine-rich α helix having 11aa (three turns of helix) (AFP Type 1) and a 3 aa repeat of Ala-Ala-Thr (side-chain disaccharide) motif (AFGP). Additional structural information can be obtained from Jia et al.

**Table 1: Structural Characteristics of AFPs**

| Characteristic | APGP (Anti-Freeze Glycoprotein) | AFP Type I | AFP Type II | AFP Type III | AFP Type IV |
|---|---|---|---|---|---|
| MW | ~2,600-33,000 | ~3,300-4,500 | ~11,000-24,000 | ~6,500 | ~12,200 |

| | | | | | |
|---|---|---|---|---|---|
| | | | 24,000 | | |
| 1° Structure | (ala-ala-thr)n disaccharide | ala rich repeats – typically 11 aa repeats | cystine rich disulphide linked | general | 17% gluramine; lack of disulphide bridges |
| Carbohydrate Linked | Yes | No | Generally not | No | No |
| 2° Structure | expanded | alpha helical amphiphilic | beta sheet | beta sandwich | amphipathic alpha helix |
| 3° Structure | NA | 100% helix | NA | NA | Four-helix Antiparallel Bundle |
| Biosynthesis | Multi-protein | Prepro AFP | Prepro-AFP | Pro-AFP | No Post-translational Modifications |
| Protein Components | 8 | 7 | 2-6 | 12 | 1 |
| Gene Copies | NA | 80-100 | 15 | 30-150 | NA |
| Natural Source | Antarctic Notothenioids; Northern Cod | Right-eyed Flounders; Sculpins | Sea raven; smelt; herring | Ocean pout; Wolffish | Longhorn Sculpin |

The present invention provides compositions and methods for enhancing signal amplification in amplification reactions and preferably in real-time PCR, as well as for increasing the freeze/thaw stability of buffer solutions involved in real-time PCR. In one embodiment, standard real-time PCR solutions of the invention generally include probe, primers, template DNA, dNTPs and nucleic acid polymerase, for example Taq DNA polymerase, and includes AFP. In preferred embodiments, the AFP is selected from the group having either an alanine-rich motif, for example, AFP type I or AFGP. In addition, fragments of AFP including the 11aa analine-rich α helix motif derived from AFP type I (see for example U.S. Patent No. 5,925,540, the disclosure of which is expressly incorporated herein by reference), or repetitive repeats of -Ala-Ala-Thr-

(disaccharide) derived from AFGP, and variants, derivatives, isoforms and fusion proteins thereof (all these examples are referred to generally as AFP for purposes of the invention) are useful in these regards. In preferred embodiments the real-time PCR buffer further includes a carrier protein, for example BSA or gelatin, to enhance the AFP derived results.

Embodiments in accordance with the present invention typically include a solution of AFP from about 10-200 μg/ml, and preferably from 25-100 μg/ml and most preferably from about 40-60 μg/ml. Inclusion of carrier protein with the AFP is typically at a concentration of about 100 μg/ml to about 300 μg/ml, and preferably from about 150 to about 250 μg/ml, and most preferably about 200 μg/ml. Inclusions of AFP and carrier protein in standard real-time PCR buffers provide a significant improvement in signal amplification as measured by average RFU, and cycle of detection as measured by threshold cycle. Taken together, inclusion of AFP alone or AFP with a carrier protein dramatically improves the signal strength of amplification assays and in particular real-time PCR assays.

AFP for use in the present invention can be purchased from A-FP Proteins, Inc., Waltham, MA. In addition, AFP, and fragments, derivatives and fusion proteins of AFP in accordance with the invention can be expressed in insect, yeast, prokaryote, and eukaryote cells. Suitable prokaryotic hosts to be used for expression include but are note limited to bacteria of the genera *Escherichia*, *Bacillus* and *Salmonella*. AFP sequence for design of recombinant protein expression can be obtained from a review of Jia et al which is and was previously incorporated herein by reference in its entirety (also see US Patent No. 5,925,540, also incorporated in its entirety).

Modifications of the amino acid sequence of AFP molecules useful in the present invention can be accomplished by any of a number of known techniques. For example, mutations may be introduced at particular locations by oligonucleotide-directed mutagenesis (Walder et al., 1986, Gene, 42:133; Bauer et al., 1985, Gene 37:73, Craik, 1985, Biotechniques, 12-19; Smith et al., 1981, Genetic Engineering: Principles and Methods, Plenum Press; US Patent No. 4,737,462). Modifications may be useful in the enhancement of AFP activity as discussed herein and shown in the Examples.

AFP polypeptides of the present invention are preferably used in an isolated or partially isolated form. The polypeptides may be recovered and purified from recombinant cell cultures by known

methods, including, for example, ammonium sulfate or ethanol precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography and lectin chromatography.

AFP polypeptides can be fused to heterologous polypeptides to facilitate purification. Many available heterologous peptides allow selective binding of the fusion protein to a binding partner. Non-limiting examples of peptide tags include 6-His, thioredoxin, hemagglutinin, GST, and the OmpA signal sequence tag. A binding partner that recognizes and binds to the heterologous peptide can be any molecule or compound, including metal ions, antibodies, antibody fragments, or any protein or peptide that preferentially binds the heterologous peptide to permit purification of the fusion protein.

The present invention further provides real-time PCR buffers having enhanced stability for use after numerous freeze-thaw cycles, and extended storage times from 4°C to -80°C (see Examples below). As above, standard amplification buffer ingredients are mixed with AFP or with AFP and a carrier protein, using the concentrations discussed above, to minimize buffer or enzyme degradation between any two slow freeze/thaw cycles. Note that the effectiveness of AFP inclusion in stabilizing PCR buffers is related to slow freeze events, for example freezing a solution between the temperatures of 0°C and -40°C.

As such, and without being bound by theory, it is believed that the inclusion of AFP or AFP in combination with a carrier protein results in the dual benefit of enhancing the sensitivity and signal strength of the real-time PCR assay, and of providing additional stability to the real-time PCR buffer and enzymes during repetitive freeze-thaw cycles. Typically, stability is provided for freeze conditions between 0°C to about -40°C. Kits having Real-Time PCR buffer that include appropriate amounts of AFP can be packaged to maximize the sensitivity of the assay, and stability storage of the buffer under slow-freeze conditions. In addition, AFP or AFP and carrier protein can be packaged with a target polymerase for addition to a PCR or other like amplification buffer.

Note that although inclusion of AFP in PCR buffers has been shown to enhance real-time PCR sensitivity and PCR and real-time PCR buffer storage stability, it is also envisioned to be useful in the inclusion of storage solutions having other enzyme activities. For example, useful in the

inclusion of any solution that will undergo slow freezing conditions that also requires the maintenance of enzyme activity, i.e., where the enzyme may be damaged by the freeze/thaw event. In one alternative embodiment, AFP, in the presence or absence of a carrier protein, is combined with AMLV to protect the reverse transcriptase activity of the enzyme over a period of several slow freeze-thaw cycles.

**Method For Enhanced Amplification Activity After One Or More Freeze/Thaw Events**

The present invention provides methods for maintaining the functional stability of an enzyme in a solution, preferably a polymerase in a PCR or real time-PCR buffer. Initially, a polyol, AFP or AFP with carrier protein is combined with a target enzyme activity. The combination can occur in a standard amplification buffer or buffers consistent with embodiments of the present invention. The combined enzyme-polyol, enzyme-AFP, or enzyme-AFP-carrier protein is utilized in the reaction. The remaining material, when frozen, is protected from some or all of the freeze/thaw effects. The enzyme mix can be used over the course of numerous freeze/thaw events. In a preferred embodiment, the amplification reaction is a real time-PCR reaction. In another embodiment, the method incorporates an enzyme mixed with at least two of: polyol, AFP, carrier protein, and most preferably all three components are included. In an additional embodiment, dNTP analog is included in the amplification reaction mix to enhance efficiency of the reactions by lowering the formation of primer-dimers.

**Compositions Of High Performance Real Time PCR Buffers**

The present invention further provides high performance amplification buffers for use in amplification reactions, preferably in standard PCR and real-time PCR. Buffers in accordance with the present invention can include sorbitol, anti-freeze protein (AFP1, AFGP, mixtures of AFP1 and AFGP), carrier protein, dNTP mix, nucleic acid polymerase, preferably a thermophilic or hyperthermophilic polymerase, and have a modified pH, obtained through a buffer system that utilizes a zwitterionic formulation. Illustrative zwitterionic formulations include HEPES-KOH KCL, TAPS K GLUT, HEPES-tris KCL, HEPES-KOH K GLUT, TAPS-KOH KCL or TAPS-tris KCL. Preferred embodiments of the present invention utilize a buffer system that includes TAPS-KOH KCL and/or TAPS-tris KCL, and most preferably TAPS-tris KCL. Preferred pH ranges for these buffers are dependent on the final use, for example, buffers for use in real-time

PCR are buffered to have a pH of from about 7.9 to about 8.7, and preferably from about 8.2 to about 8.7. Note that buffers for use in standard PCR are modified to have a pH of from about 7.9 to 8.9. In preferred embodiments, the dNTP mix of the buffer system includes from about 10% to about 50% dUTPs (in replacement of dTTPs in the dNTP mix), and more preferably from about 10% to about 30% dUTPs (in replacement of dTTPs in the dNTP mix). Further, in some embodiments, DMSO, SSBP, n-propyl sulfoxide, and/or trehalose can be included in the high performance buffer.

Concentrations of ingredients useful in embodiments of the high performance buffer are as shown in Table 2. Note that the preferred concentration for TAPs-KOH KCL is 150mM and for the TAPs-tris KCL is 500mM, both with a final buffer pH of about 8.

**Table 2: High Performance PCR Buffer/Real-Time PCR Buffer**

| **Buffer Ingredient** | **Useful Concentration Range (Final)** | **Preferred Concentration (Final)** |
|---|---|---|
| Sorbitol, Trehalose, DMSO and/or mixtures thereof | 10mM – 300mM | 100mM |
| dNTP Mix/% dUTP in Replacement of dTTP (or other dNTP analogs) | 400μM per dNTP | 200μM per dNTP |
| Nucleic Acid Polymerase | 0.5 to 2 Units | 2 Units |
| Mg Ion Concentration | 3mM to 10mM | 5mM and 8mM |
| Potassium Salt Concentration | 30mM to 80mM | 40mM to 60mM |
| anti-freeze protein, e.g., AFP type I, AFGP or mixtures of same//Carrier Protein | 10μg/ml to 200μg/ml//100μg/ml to 300μg/ml | 50μg/ml//100μg/ml |

| | | |
|---|---|---|
| Buffering Ingredient, e.g., TAPS-tris KCL | 100mM to 500mM | 150mM to 500mM |

The present invention further provides the AFP zwitterionic buffer compositions discussed above having a monovalent potassium salt ranging in concentration from about 30mM to about 80mM, and preferably between about 40mM and 60mM. Additionally, magnesium ions in buffers of the present invention can be from about 2.5mM to about 10mM , and preferably from about 5mM and about 8mM.

It is also envisioned that embodiments using modified dNTP mixtures to limit primer-dimer formation in accordance with embodiments previously described can be included with embodiments of the high performance PCR buffers of the invention. As such, embodiments using the zwitterionic buffer formulations can have one or more of AFP, carrier protein, sorbitol, mannitol, DMSO, SSBP, and a dNTP mix having a percentage of the dTTP or other dNTP replaced with a dNTP analog like dUTP. Typically, the composition has a pH of between about 7.9 and 8.2 for optimal effects. Other pH can be used but with limited results.

**PCR and Real-Time PCR Buffer Kits**

The present invention further provides kits that include the composition embodiments of the present invention. Kits can include PCR buffers of the invention, for example embodiments of the high performance PCR buffers of the invention, or alternatively, pre-determined stand alone amounts of AFP, sorbitol, mannitol, or other compositions of the invention, which are added to PCR buffers or are combined with enzymes used in the target use, combined with the invention. In addition, kit compositions can include combinations of AFP with different polymerase enzymes in the same or different tubes.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

**EXAMPLES**

**Example 1: dUTP Incorporation During Amplification Reactions Reduces Primer-Dimer Formation**

Replacement of dTTP with dUTP during amplification reactions reduces primer-dimer formation where the primer has a 5' overhang. A series of PCR reactions were prepared having GADPH and SRY forward and reverse primers, GADPH and SRY probes, and appropriate plasmid templates. Each reaction either received standard dNTP mixes (10 mM of dATP, dCTP, dGTP and dTTP) or modified dNTP mixes where the dATP, dCTP and dGTP were held constant at 10 mM and some or all of the dTTP was replaced with dUTP. For example a 20% dUTP reaction contained 10 mM of dATP, 10 mM dCTP, 10 mM dGTP, 8 mM dTTP and 2 mM dUTP.

As shown in Figure 1, primer-dimer formation is significantly reduced through inclusion of dUTP into the PCR reaction conditions. dUTP replacement seems to have a maximal effect on reducing dimer formation when approximately 50% of the dTTP is replaced with dUTP.

As shown in Figure 2, inclusion of dUTP into the PCR reaction mixture causes a dramatic decrease in primer-dimer formation. Both HotMaster Taq and NTC supported the decrease in primer-dimer formation. Tables 3 and 4 illustrate the reaction conditions used for a three step thermal cycle - 95°C for one minute – then for twenty seconds, step 2, 53°C for twenty seconds and 68°C for twenty seconds.

**Table 3: HotMaster Taq Reaction With Standard dNTPs**

| Reaction Component | Initial Concentration | Final Concentration Volumes |
|---|---|---|
| QuantMaster Probe Buffer **(Source)** | 10X | 1X |
| dATP | 10mM | 200µM |
| dCTP | 10mM | 200µM |
| dGTP | 10mM | 200µM |

| | | |
|---|---|---|
| dTTP | 10mM | 200μM |
| Factor VIII Forward Primer | 10μM | 200nM |
| Factor VIII Reverse Primer | 10μM | 200nM |
| Taq Polymerase | 5U/μM | 1U/μM |
| MBGW | NA | 36.8-38.8μl |
| Humand gDNA (Promega) | 25ng/μl | 50 ng |

**Table 4: HotMaster Taq Reactions With dUTP Mix**

| Reaction Component | Initial Concentration or Volume | Final Concentration Volume |
|---|---|---|
| QuantMaster Probe Buffer | 10X | 1X |
| dATP | 10mM | 200μM |
| dCTP | 10mM | 200μM |
| dGTP | 10mM | 200μM |
| dTTP | 8mM | 160μM |
| dUTP | 2mM | 40μM |
| Factor VIII ForwardPrimer | 10μM | 200nM |
| Factor VIII Reverse Primer | 10μM | 200nM |
| Taq Polymerase | 5U/μl | 1U |

| | | |
|---|---|---|
| MBGW | NA | 36.8-38.8µl |
| human gDNA (Promega) | 25 ng/µl | 50 ng |

The preceding Example illustrates that replacement of up-to 50% or more of the dTTP with dUTP results in a reduction in primer-dimer formation, thereby maximizing the specificity of the reaction conditions.

**Example 2: dNTP Analogs Shift The Melt Curve To Lower Temperatures**

The following Example was performed to compare the effect on template melting by replacement of a percentage of the dNTPs with a dNTP analog in the standard PCR buffer. Reactions were prepared as shown in Tables 5 and 6, and run with the appropriate analog as per protocol outlined in Table 7.

**Table 5: dNTP Analog Mix Composition**

| Analog | Standard dNTP Mix | 7-deaza-dGTP Mix | dITP Mix | dUTP Mix | dUTP/deaza Mix | dUTP/dITP | dUTP/dITP/deaza Mix |
|---|---|---|---|---|---|---|---|
| dATP | 10mM | 10mM | 10mM | 10mM | 10mM | 10mM | 10mM |
| dCTP | 10mM | 10mM | 10mM | 10mM | 10mM | 10mM | 10mM |
| dGTP | 10mM | 5mM | 5mM | 10mM | 5mM | 5mM | 5mM |
| dTTP | 10mM | 10mM | 10mM | 5mM | 5mM | 5mM | 5mM |
| dUTP | | | | 5mM | 5mM | 5mM | 5mM |
| dITP | | | 5mM | | | 5mM | 2.5mM |
| 7-deaza- | | 5mM | | | 5mM | | 2.5mM |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| dGTP | | | | | | | |

**Table 6: Reaction Set-Up For Figure 3**

| Reaction Component | Final Concentration | µL/50µL Reaction |
|---|---|---|
| 10X QuantMaster SYBR Buffer | 1X | 5µL |
| dNTP Analog Mix | see Table __ | 1.5µL |
| TNF-A Forward Primer (10µM) | 100nM | 0.5µL |
| TNF-A Reverse Primer (10µM) | 100nM | 0.5µL |
| SYBR Green I (1:5K) | 1:50K | 5µL |
| HotMaster Taq DNA Polymerase (5U/µL) | 1U | 0.2µL |
| MBGW | N/A | 36.8µL |
| Human gDNA (50ng/µL) | 25ng | 0.5µL |

**Table 7: Cycle Protocol**

| Cycle Number | Temperature | Time |
|---|---|---|
| Cycle 1 | (1X) | |

| | | |
|---|---|---|
| | Step 1 95.0°C | 1:00 |
| Cycle 2 | (40X)<br>Step 1 95.0°C<br>Step 2 58.0°C<br>Step 3 68.0°C<br>Data collection and real-time analysis enabled | <br>0:20<br>0:20<br>0:20 |
| Cycle 3 | (1X)<br>Step 1: 95°C | <br>1:00 |
| Cycle 4 | (1X)<br>Step 1: 55.0°C | <br>2:00 |
| Cycle 5 | (80X)<br>Step 1: 55.0°C<br>Increase setpoint temperature after cycle 2 by 0.5°C, melt curve data collection and analysis enabled (see Figure 3) | <br>0:10 |
| Cycle 6 | (1X)<br>Step 1: 4.0°C | <br>Hold |

Figure 3 illustrates that replacement of one or more standard dNTPs with a dNTP analog results in a small but significant facilitation of template melting as compared to standard dNTP

compositions. This data illustrates the decreased thermodynamically favorability for incorporation at primer annealing conditions

**Example 3: dUTP Does Not Effect Ct, RFU or Yield During Real Time-PCR**

From the proceeding Examples, inclusion of dUTP for dTTP provides a significant benefit toward reducing the levels of primer-dimer formation during PCR. To determine whether the replacement of dTTP with dUTP in a standard dNTP mix adversely affected sensitivity or signal size during PCR, for example, during real time PCR, comparisons were made between reactions that had from 2.5mM to 7.5mM dUTP in replacement of dTTP. Reactions were as substantially described in Example 1, except that a portion of the 10mM dTTP was replaced with either 2.5mM dUTP or 7.5mM dUTP.

As shown in Figure 4A, inclusion of dUTP for dTTP had little or no affect on either signal amplification or threshold cycle Ct. Further, inclusion of dUTP in the real-time PCR has little or no effect on the product yield of the reaction (see Figure 4B).

This Example, in combination with the data shown in Examples 1 and 2, shows the utility of the present invention for providing a PCR buffer useful in reducing primer-dimer formation while maintaining yield, signal size (RFU)and Ct cycle sensitivity.

**Example 4: Sorbitol Facilitates Removal of Template Secondary Structure**

A system was developed to investigate the effect target chemicals had on template secondary structure. As shown in Figure 5A and 5B, one template (derived from β-Actin) was provided within an amplification reaction, a first structure (shown schematically in 5A) having little or no secondary structure, and a second structure (shown schematically in 5B) having a significant portion of secondary structure. The second structure includes an extended region of GC rich sequence and differs from the first structure when amplified. In the absence of other factors, little or no amplification from the second structure is anticipated due to the extended region of secondary structure. Conversely, amplification from the first structure molecule results in significant product formation. Only when the secondary structure is removed with the second structure be amplified, thereby providing the product of that length.

Experiments were performed to determine the effect of target chemical agents on their ability to melt-out secondary structure from template structure number two. An increase in amplification of the second structure is indicative of the chemical agents ability to melt-out the secondary structure. Template was incubated in a standard PCR reaction with increasing amounts of sorbitol or increasing amounts of sorbitol in the presence of n-proyl-sulfoxide. Amplified products were visualized by running on an agarose gel and stained with ethidium bromide. Thermal cycling included an initial 95°C for sixty seconds step followed by 95°C for twenty seconds, 59°C for twenty seconds, and 68°C for twenty seconds repeated forty times. Tables 8, 9 and 10 illustrate reaction conditions for Figures 7A, 7B and 7C respectively:

**Table 8: Reaction Conditions For Figure 7A**

| Reaction Mixture | Reaction Component Volume | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rnx component | #1 | #2 | #3 | #4 | #5 | #6 | #7 | #8 | #9 | #10 | #11 |
| Master Mix* (μl) | 18.2 | 18.2 | 18.2 | 18.2 | 18.2 | 18.2 | 18.2 | 18.2 | 18.2 | 18.2 | 18.2 |
| 10mM Standard dNTP Mix (μl) | 1 | 1 | 1 | | | | | | | | |
| 25% dUTP (μl) | | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 99.9% DMSO (μl) | 1.5 | | | 1.5 | | | 1.5 | | | 1.5 | 1.5 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1M Sorbitol (µl) | | 15 | | | 15 | | | 15 | | 15 | |
| 1M Mannitol (µl) | | | 15 | | | 15 | | | 15 | | 15 |
| SSBP (150 ng/ml) (µl) | | | | | | | 1 | 1 | 1 | 1 | 1 |
| MBGW (µl) | 29.3 | 15.8 | 15.8 | 29.3 | 15.8 | 15.8 | 28.3 | 14.8 | 14.8 | 13.3 | 13.3 |
| Total (µl) | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Buffer, 100nM forward and reverse β-Actin Primer, 1U Taq polymerase, and 50ng gDNA | | | | | | | | | | | |

**Table 9: Reaction Conditions For Figure 7B**

| Set-Up Per 50µl Reaction – all values are in µl | | | | |
|---|---|---|---|---|
| Reaction Component | HM Control | 2% DMSO | 2.5% DMSO | 3% DMSO |
| 10X HotMaster Buffer | 5 | 5 | 5 | 5 |
| Taq Polymerase | 0.2 | 0.2 | 0.2 | 0.2 |
| 10mM Stnd dNTP Mix | 1 | 1 | 1 | 1 |

| | | | | |
|---|---|---|---|---|
| β-Actin Forward Primer (10μM) | 1 | 1 | 1 | 1 |
| β-Actin Reverse Primer (10μM) | 1 | 1 | 1 | 1 |
| 1M Sorbitol | 0 | 15 | 15 | 15 |
| 99.9% DMSO | 0 | 1 | 1.25 | 1.5 |
| gDNA (25ng/μl) | 2 | 2 | 2 | 2 |
| MBGW | 39.2 | 23.8 | 23.55 | 23.3 |

**Table 10: Reaction Conditions For Figure 7C**

| General Master Mix | | |
|---|---|---|
| Reaction Component | Initial Concentration | Final Concentration Volume |
| QuantMaster Probe Buffer | 10X | 1X |
| dNTP Mix | | |
| dATP | 10mM | 200μM |
| dCTP | 10mM | 200μM |
| dGTP | 10mM | 200μM |
| dTTP | 7.5mM | 150μM |
| dUTP | 2.5mM | 50μM |

| | | | | | | |
|---|---|---|---|---|---|---|
| β-Actin Forward Primer | | 10μM | | 200nM | | |
| β-Actin Reverse Primer | | 10μM | | 200nM | | |
| SSBP | | 150ng/μl | | 150ng | | |
| Taq Polymerase | | 5U/μl | | 2U | | |
| MBGW | | NA | | 10μl | | |
| gDNA | | 25ng/μl | | 50ng | | |
| Samples Containing No Trehalose | | | | | | |
| Reaction Component | 0mM Sorbitol | 10mM Sorbitol | 40mM Sorbitol | 100mM Sorbitol | 200mM Sorbitol | 300mM Sorbitol |
| MBGW/50μl reaction | 30 | 29.5 | 28 | 25 | 20 | 15 |
| 1M Sorbitol | 0 | 0.5 | 2 | 5 | 10 | 15 |
| Samples Containing 300mM Trehalose | | | | | | |
| MBGW | 25 | 25 | 25 | 25 | 25 | 25 |
| 1M Sorbitol/Tehalose Mixture | 5 | 5 | 5 | 5 | 5 | 5 |
| HotMaster Control Reactions | | | | | | |
| Reaction Component | | Initial Concentration | | Final Concentration | | |
| HotMaster Buffer | | 10X | | 1X | | |

| | | |
|---|---|---|
| Standard dNTP Mix | 10mM Each | 200μM each |
| β-Actin Forward Primer | 10μM | 200nM |
| β-Actin Reverse Primer | 10μM | 200nM |
| SSBP | 150ng/μl | 150ng |
| HotMaster Taq Polymerase | 5U/μl | 2U |
| MBGW | NA | 10μl |
| gDNA | 25ng/μl | 50ng |

Figures 6, 7A, 7B and 7C illustrate that sorbitol alone or in combination with other agents, for example n-propyl sulfoxide, dUTP, DMSO, mannitol and single-stranded binding protein facilitated the amplification of the longer, often GC rich, template by reducing the amount of secondary structure within the template. Note that combinations of increasing amounts of sorbitol and 7% n-propyl sulfoxide (Figure 6) and 300nM sorbitol and 2-3% DMSO were particularly effective in melting out the secondary structure and allowing for amplification of the longer template. Note also that increasing amounts of sorbitol in the presence of trehalose (right panel of Figure 7C) caused a preferential amplification of the larger, secondary structure containing, plasmid.

The preceding Example illustrates the utility of including sorbitol alone or in combination with trehalose, n-propyl sulfoxide, dUTP, DMSO, mannitol and SSBP, for amplification of template DNA, and in particular, template DNA high in secondary structure.

**Example 5: Sorbitol Enhances Stability of Real Time-PCR Master Mix During Extended Periods of Storage at -20°**

Sorbitol was included in real time-PCR master mix to determine its effects on storage stability of real time-PCR master mix buffers. Master mix samples were prepared with and without 100mM

sorbitol, stored at 4, -20 or –80° C for one, two or three weeks and tested for overall performance in real time-PCR. Real time-PCR components were as described above in Example 4.

As shown in Figures 8A and 8B, 100mM sorbitol provided for an earlier Ct for reactions conducted with master mix buffers frozen at -20°C for one, two or three weeks (8A). Corresponding RFU values were also maintained in the sorbitol containing master mixes at this temperature. Samples stored at either 4°C or -80°C showed smaller or no change between the sorbitol containing and non-containing samples. It is believed that sorbitol is most effective at stabilizing master mix storage at -20°C due to the slow freeze nature of this temperature range. In comparison, storage of the master mix at either 4°C or -80°C was not expected to provide the same protection, as 4°C does not freeze samples and -80°C is a very fast snap freeze, less likely to involve formation of damaging water crystals, although as shown below some benefit is obtained.

Figures 9A and 9B provide corresponding yield comparisons for one and three week storage, again indicating the usefulness of sorbitol in stabilizing the master mix at -20°C in RT-PCR. Note that the yield is significantly lowered when no sorbitol is included in the master mix stored at -20°C (lane 8 in 9A and lane 5 in 9B). However, a dramatic increase for corresponding samples occurs when 100mM sorbitol is included in the master mix buffers.

**Example 6: Anti-Freeze Proteins (AFPs) Enhance Stability of Real Time-PCR Master Mix During Extended Periods of Storage at -20°**

Given the impressive results of sorbitol enhancing the freeze-thaw stability of real-time PCR products, the effects of AFP1 on the freeze-thaw stability of real-time PCR buffers was investigated. Initially, AFP1 was titrated into real time-PCR reactions to determine what concentration of AFP1 did not detrimentally effect yield of RT-PCR product formation. Real-time PCR was performed as described in Examples above, and products visualized on ethidium bromide stained 1% agarose gels. As shown in Figure 10, product yields were not detrimentally effected until approximately 100 μg/ml AFP1 was included in the reaction mix. Conversely, addition of 50μg/ml AFP1 to the real time-PCR had little or no effect on the reaction yields (compare the lane having 0 AFP1 to the lane having 50μg/ml AFP1).

Prior to determining the freeze-thaw capacity of Real-Time PCR buffers in the presence and absence of AFP1, the ability of AFP1 to enhance fresh Real-Time PCR samples was tested. Real-Time PCR was prepared as above and samples incubated with either 200 μg/ml BSA, 50 μg/ml AFP1, or 200 μg/ml BSA mixed with 50 μg/ml AFP1. Figures 11A and B graphically show that the addition of AFP1 alone actually has a slightly negative effect on the average RFU (A) and Threshold cycle (B). BSA alone had little or no effect on real-time PCR. However, combination of BSA and AFP1 provided marked improvement on both signal amplification (A) and threshold cycle (B). As such, the combination of AFP1 and a carrier protein like BSA provides substantial improvement during real-time PCR (while maintaining maximal yield of the amplicons – see Figure 10).

AFP1:BSA containing real-time PCR samples were next tested for stability during freeze-thaw conditions over zero (Figures 11 and 12), one week (Figure 12), two weeks (Figure 12) and three weeks (Figure 12) at -20°C. Real-time PCR conditions were as described above except for the inclusion of the different amounts of carrier protein and AFP1. As shown in Figure 12A and B, the enhanced signal obtained during Real-Time PCR was maintained when AFP1 and BSA were added to the reaction mix (A) and the threshold cycle was also maintained as compared to the changes noted in BSA alone, AFP1 alone or no additives (B).

Similar Real Time PCR studies were performed using AFGP in the presence and absence of a carrier protein to determine whether the AFP1 effects were limited to AFP1 or were extendable to other AFP family members, and particularly to AFP family members having similar primary and secondary protein structure. AFGP includes a primary and secondary protein structure similar to AFP1 – particularly, strong similarity exists between the alanine-rich motif having an alpha helical amphiphilic secondary structure (see Table 1) and the alanine-alanine-threonine repeat disaccharide of AFGP. Like AFP1, AFGP in combination with BSA, had a significant effect on maintaining or lowering threshold cycle number as compared to control samples. Experimental parameters are shown in Table 11 below.

**Table 11: AFP1 and AFGP Reaction Set-up**

| **Buffer Master Mix for PCR** |
|---|

| Component | Stock | Final | µL/20µL rxn | # reactions | nuclease free water |
|---|---|---|---|---|---|
| 10X PCR buffer (1M sorbitol) | 10 | 2.5 | 5 | 800 | ---- |
| dNTP with UTP mix (mM) | 10 | 1 | 2 | 320 | ---- |
| glycerol (%) | 100 | 1 | 0.2 | 32 | ---- |
| DNA poly (U/µL) (Eppendorf Hot Master) | 5 | 2 | 0.4 | 64 | ---- |
| Total | | | 7.6 | 1216 | |
| Aliquot Master Mix into 4 tubes 167.2µL (22 rxns) each | | | | | |
| **Buffer Master Mix With BSA** | | | | | |
| BSA (mg/ml) | 50 | 0.5 | 0.2 | 4.4 | 268.4 |
| **Buffer Master Mix With AFGP** | | | | | |
| AFGP (mg/ml) | 1 | 0.125 | 2.5 | 55 | 217.8 |
| **Buffer Master Mix With BSA and AFP1** | | | | | |
| BSA (mg/ml) | 50 | 0.5 | 0.2 | 4.4 | 213.4 |

| | | | | | |
|---|---|---|---|---|---|
| AFP1 (mg/ml) | 1 | 0.125 | 2.5 | 55 | ---- |
| **Buffer Master Mix No Additives** | | | | | |
| No Additive Control | 0 | 0 | 0 | 0 | 272.8 |
| **Buffer Master Mix With BSA and AFGP** | | | | | |
| BSA (mg/ml) | 50 | 0.5 | 0.2 | 4.4 | 213.4 |
| AFGP (mg/ml) | 1 | 0.125 | 2.5 | 55 | ---- |
| **Buffer Master Mix With BSA, AFP1, AFGP** | | | | | |
| BSA (mg/ml) | 50 | 0.5 | 0.2 | 4.4 | 158.4 |
| AFGP (mg/ml) | 1 | 0.125 | 2.5 | 55 | ---- |
| AFP1 (mg/ml) | 1 | 0.125 | 2.5 | 55 | ---- |
| **Buffer Master Mix With AFP1, AFGP** | | | | | |
| AFP1 (mg/ml) | 1 | 0.125 | 2.5 | 55 | ---- |
| AFGP (mg/ml) | 1 | 0.125 | 2.5 | 55 | ---- |

As shown in Figure 16, AFGP showed similar results in threshold cycle for fresh and freeze-thawed samples (-20°C) for one, two or three weeks. Note that the combination of AFP type I,

AFGP and BSA provided the best overall performance during real time-PCR stability testing, as compared to the controls but also as compared to AFP type I alone, AFGP alone, AFP type I with BSA and AFGP with BSA.

The results in this Example show the utility of including AFP1 and AFGP, in the presence of a carrier protein, in Real-Time PCR reactions for facilitating the stability of the buffer during multiple freeze-thaw events at -20°C. This Example also provides data that at least two anti-freeze protein family members supported long term stability of a Real Time-PCR buffer, AFP type I and AFGP. The data provides strong evidence that proteins that exhibit similar anti-freeze protein like functional properties will be effective in similar manners to these proteins, and that the functional motif of AFP type I and AFGP (see Table 1 above) will be effective in a similar manner.

**Example 7: pH modification and Buffer Type Have Significant Effect on Optimizing Sorbitol, AFP1 Containing Real-Time PCR Buffer**

Several real time-PCR buffer combinations were tested for signal amplification and for threshold cycle in the presence of a standard amount of both sorbitol and AFP1/BSA. Both the buffering component and salt were modified to provide different ionic concentrations and pHs. In particular, HEPES-KOH KCL, 150 mM, pH 8; TAPS K GLUT, 150 mM, pH 8; HEPES-tris KCL, 500 mM, pH 8; Bicine-tris K GLUT pH 8.7; HEPES-KOH K GLUT, 150 mM, pH 8; Bicine-tris K GLUT, 500 mM, pH 8; TAPS-KOH KCL, 150 mM, pH 8; Bicine-tris KCL, 150 mM, pH 8; TAPS-tris KCL, 500 mM, pH 8; and RealMaster 10X standard buffer (embodiment of the present invention) were compared for ability to support highly accurate real time-PCR (each buffer also included 50μg/ml AFP1, 200μg/ml BSA and 100 mM sorbitol).

As shown graphically in Figure 13A and B (and Table 12), buffers composed on TAPS-KOH KCL and TAPS-tris KCL provided the lowest threshold cycle and highest signal amplification in Real-Time PCR. In both cases the buffers significantly outperformed the RealMaster 10X standard buffer.

**Table 12: Real-Time PCR Buffer Test Data For Alternative Buffer Constituents**

| Buffer Type | Ave Ct | Standard Deviation of Ct | Ave RFU | Standard deviation of RFU |
|---|---|---|---|---|
| HEPES-KOH KCL 150mM, pH8 | 18.86667 | 0.057735 | 1751.46 | 90.14655 |
| TAPS K GLUT 150mM, pH8 | 18.83333 | 0.11547 | 2037.563 | 64.23551 |
| HEPES-tris KCL 500mM, pH8 | 18.63333 | 0.057735 | 1873.903 | 32.29925 |
| Bicine-tris K GLUT, pH8.7 | 19.7 | 0.173205 | 1308.253 | 99.58234 |
| HEPES-KOH K GLUT 150mM, pH8 | 19.0333 | 0.11547 | 1691.167 | 78.98864 |
| Bicine-tris K GLUT pH8 | 19.2333 | 0.057735 | 1666.757 | 31.04646 |
| TAPS-KOH KCL 150mM, pH8 | 18.4333 | 0.057735 | 2243.12 | 29.99653 |
| Bicine-tris KCL, pH8 | 18.8 | 0.1 | 1786.747 | 53.66013 |
| TAPS-tris KCL 500mM, pH8 | 18.36667 | 0.057735 | 2110.447 | 77.15043 |
| RealMaster 10X standard pH 8.4 | 19.1 | 0.1 | 1489.81 | 28.70814 |

| | | | | |
|---|---|---|---|---|
| standard pH 8.4 | | | | |

Further characterization was made on the buffer compositions by comparing the TAPS-KOH KCL, 150mM, pH8 , TAPS-tris KCL, 500mM, pH8 and RealMaster 10X standard buffer, pH 8.4 for ability to support Real-Time PCR. Results shown in Figure 14 graphically illustrate RFU and threshold cycle for each buffer condition, showing that TAPS-tris KCL, 500mM (pH8) out-performed both the TAPS-KOH and RealMaster 10X standard buffer. Finally, results shown in Figure 15 illustrate that TAPS-tris KCL, 500 mM, pH 8 outperformed both RealMaster 10X standard buffer, pH 8.4, and Invitrogen Platinum qPCR Supermix-UDG (Invitrogen product number 11730-017). Real-Time PCR conditions for the RealMaster 10X standard buffer and Invitrogen Platinum qPCR buffers were performed using manufacturer recommended conditions.

This data shows that a Real-Time PCR buffer composed of TAPS-tris KCL, pH of about 8, and including sorbitol, AFP1 and BSA, provided significant improvement in both signal size and threshold cycle over RealMaster and Invitrogen based products. These same buffers also help stabilize buffer storage at -20°C for longer term storage needs of the investigator.

**Example 8: AFP Containing PCR Master Mix Functionally Outperforms Competitor PCR Mix**

An embodiment of a PCR Master Mix prepared in accordance with the present invention was functionally compared to Invitrogen Platinum qPCR Buffer for its ability to support long term stability within real time-PCR. Long term stability test experimental parameters are shown in Table 13. Cycling parameters included: 95°C for one minute and forty cycles of 95°C for twenty seconds, 56°C for ten seconds and 68°C for thirty seconds.

**Table 13: Long Term Stability Test**

| Aliquot 70µl of each of the RM, 2.5X master mix into a biopure tube | | | | |
|---|---|---|---|---|
| Component | Stock | Final | µl/50µl rxn | 3.5 |

| | | | | |
|---|---|---|---|---|
| 2.5X Real Master Mix +/- ROX(x) | 2.5 | 1 | 20 | 70 |
| **Primer-Probe Template Mix For 2.5X MM** | | | | |
| B2M fwd primer (μM) | 10 | 0.2 | 1 | 22 |
| B2M rev primer (μM) | 10 | 0.2 | 1 | 22 |
| B2M FAM probe (μM) | 7.5 | 0.15 | 1 | 22 |
| gDNA male (50ng/μl) | 50 | 1 | 1 | 22 |
| MBGW | | | 26 | 572 |
| Total | | | 30 | 660 |
| Add 105μl (3.5 rxn) of primer-probe-template mix to each aliquot of 2.5X master mix. Aliquot 87.5μl of each of the competitors 2X master mixes into a biopure tube. | | | | |
| 2X Invitrogen PCR probe Mix | 2 | 1 | 25 | 87.5 |
| **Primer-Probe-Template Mix For 2X MM** | | | | |
| B2M fwd primer (μM) | 10 | 0.2 | 1 | 33 |
| B2M rev primer (μM) | 10 | 0.2 | 1 | 33 |

| | | | | |
|---|---|---|---|---|
| (μM) | | | | |
| B2M FAM probe (μM) | 7.5 | 0.15 | 1 | 33 |
| gDNA male (50ng/μl) | 50 | 1 | 1 | 33 |
| MBGW | | | 21 | 693 |
| Total | | | 25 | 825 |
| Add 87.5μl (3.5 rxns) of primer-probe-template mix to each aliquot of 2X master mix | | | | |

As shown in Figure 17, the AFP containing Real Time-PCR buffer of the present invention supported stronger signal amplification (RFU) and earlier Ct at -4°C, -20°C and -80°C as compared to Invitrogen Platinum qPCR Buffer over a six week course of experiments. The data illustrates that the buffers of the present invention provide comparable or better results for long term stability, comparable to buffers sold as state of the art. As such, embodiments of the present invention have utility in providing long term buffer stability useful in real time PCR. Note that Invitrogen product was purchased and used per manufacturers recommendations.

The invention has been described with reference to specific examples. These examples are not meant to limit the invention in any way. It is understood for purposes of this disclosure, that various changes and modifications may be made to the invention that are well within the scope of the invention. Numerous other changes may be made which will readily suggest themselves to those skilled in the art and which are encompassed in the spirit of the invention disclosed herein and as defined in the appended claims.

This specification contains numerous citations to patents, patent applications, and publications, each is hereby incorporated by reference for all purposes.

## CLAIMS

**What is Claimed is:**

1. An improved nucleic acid amplification reaction mixture, said mixture comprising at least one of a polyol, an anti-freeze protein and a dUTP/dTTP combination.

# METHODS AND COMPOSITIONS TO ENHANCE AMPLIFICATION EFFICIENCY AND SIGNAL

## Abstract of the Disclosure

Methods and compositions are provided for enhanced specificity, sensitivity, signal size, and storage stability of standard PCR buffers, real-time PCR buffers or both. Buffers in accordance with the present invention can include dNTP mixtures wherein a portion of the dTTPs has been replaced with dUTP or other like analog, and also includes sorbitol, trehalose, DMSO, n-propyl sulfoxide alone or in combination with each other , anti-freeze protein(s), optionally with a carrier protein, and is pH to about 8.0 to 8.1 in a TAPS-tris KCL or TAPS-KOH KCL buffer.

# BEST AVAILABLE IMAGES

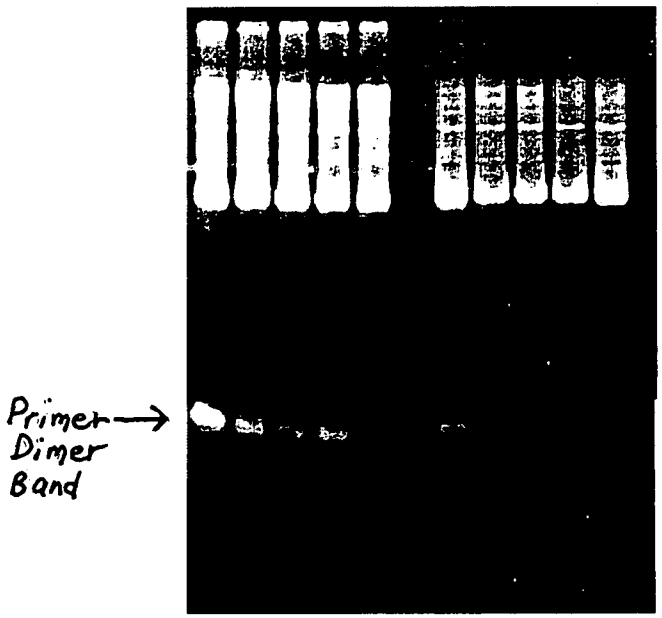
Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

## IMAGES ARE BEST AVAILABLE COPY.

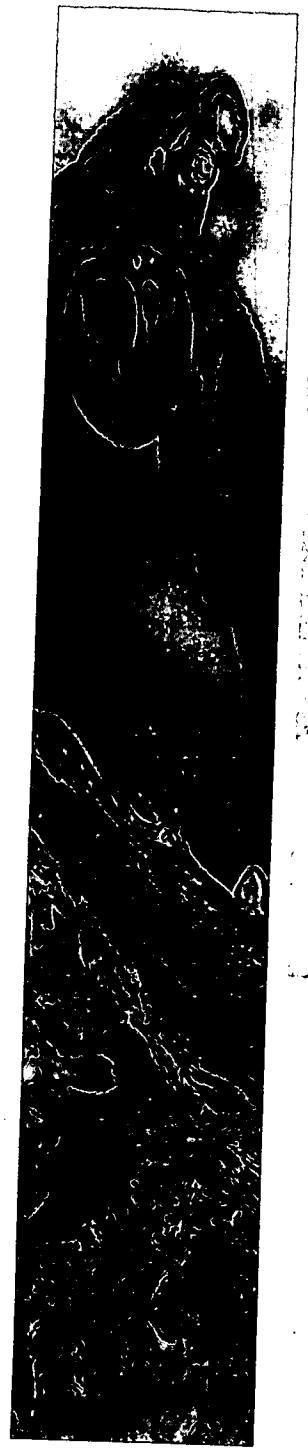
**As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.**



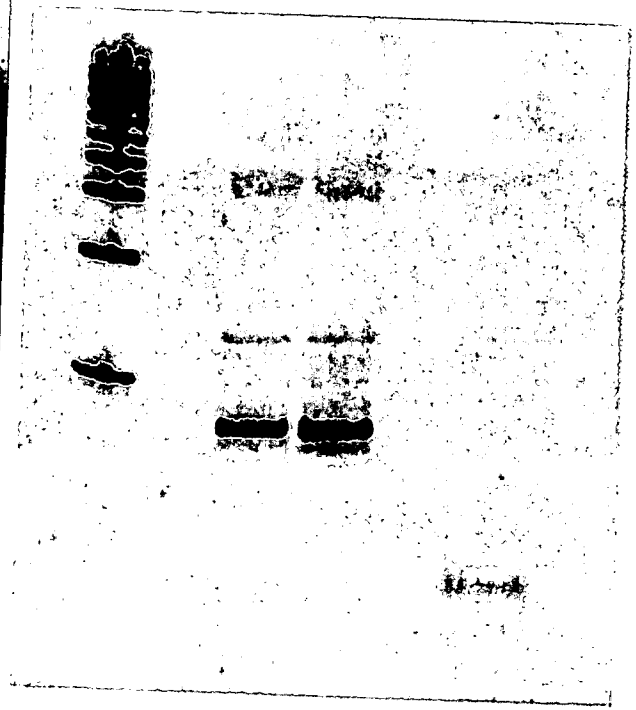

eppendorf

*In touch with life*

Figure 1



[redacted]



100bp Ladder

HotMaster Taq w/ STND dNTPs

HotMaster Taq w/ 20% dUTP Mix

NTC w/ STND dNTPs

NTC w/ 20% dUTP Mix

**Protocol**

| | | | |
|---|---|---|---|
| Cycle 1: (1X) | | | |
| | Step 1: | 95.0 C | for 01:00 |
| Cycle 2: (40X) | | | |
| | Step 1: | 95.0 C | for 00:20 |
| | Step 2: | 53.0 C | for 00:20 |
| | Step 3: | 68.0 C | f r 00:20 |
| Cycle 3: (1X) | | | |

HotMaster Taq Reactions w/ STND dNTPs

| Reaction Component | Initial Concentration or Volume | Final Concentration/ Volume |
|---|---|---|
| QuantMaster Probe Buffer | 10X | 1X |
| dNTP Mix | | |
| dATP | 10mM | 200uM |
| dCTP | 10mM | 200uM |
| dGTP | 10mM | 200uM |
| dTTP | 10mM | 200uM |
| FactorVIII Forward Primer | 10uM | 200nM |
| FactorVIII Reverse Primer | 10uM | 200nM |
| HotMaster Taq Polymerase | 5U/ul | 1U |
| MBGW | N/A | 36.8 - 38.8 uL |
| *Human gDNA (Promega) | 25ng/uL | 50ng |

* Not included in NTCs

HotMaster Taq Reactions w/ 20% dUTP Mix

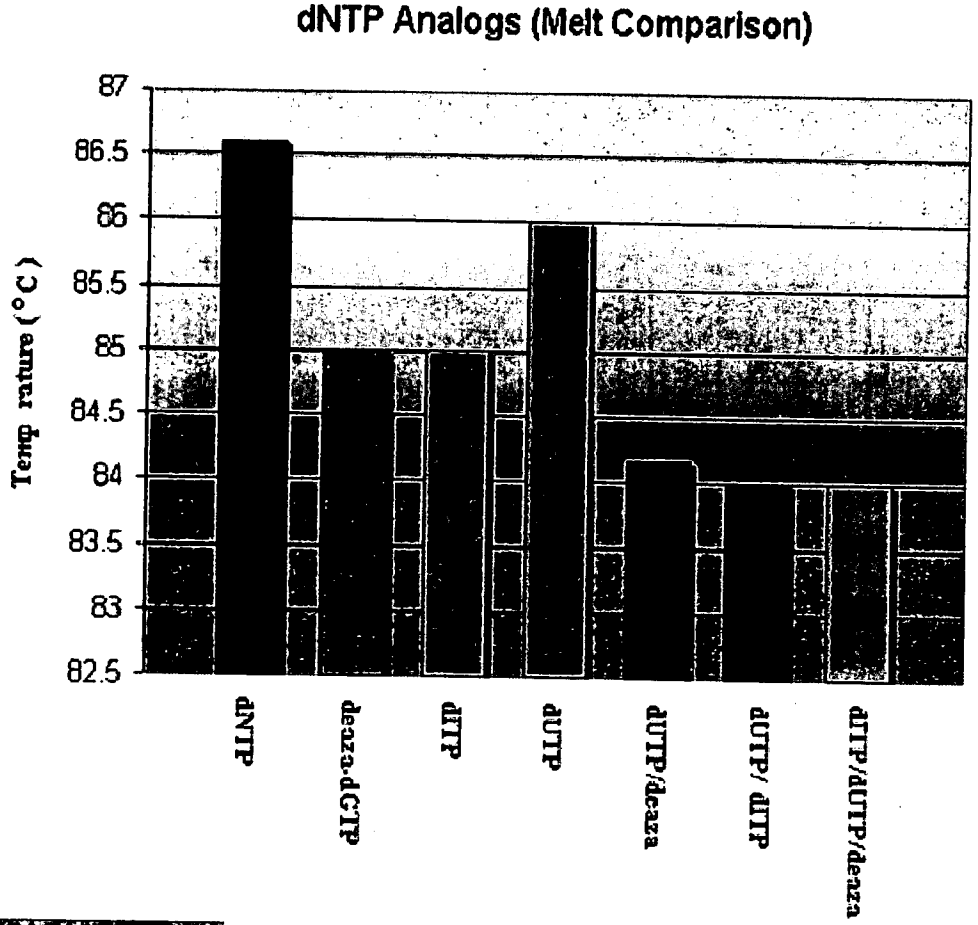
| Reaction Component | Initial Concentration or Volume | Final Concentration/ Volume |
|---|---|---|
| QuantMaster Probe Buffer | 10X | 1X |
| dNTP Mix | | |
| dATP | 10mM | 200uM |
| dCTP | 10mM | 200uM |
| dGTP | 10mM | 200uM |
| dTTP | 8mM | 160uM |
| dUTP | 2mM | 40uM |
| FactorVIII Forward Primer | 10uM | 200nM |
| FactorVIII Reverse Primer | 10uM | 200nM |
| HotMaster Taq Polymerase | 5U/ul | 1U |
| MBGW | N/A | 36.8 - 38.8 uL |
| *Human gDNA (Promega) | 25ng/uL | 50ng |

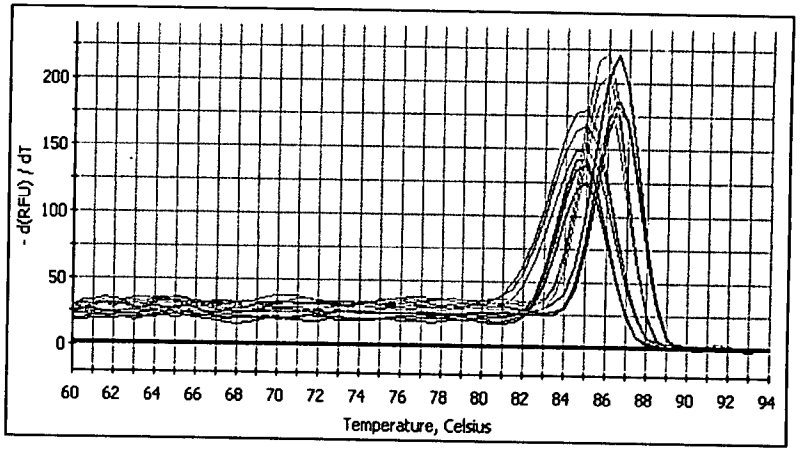
* Not included in NTCs

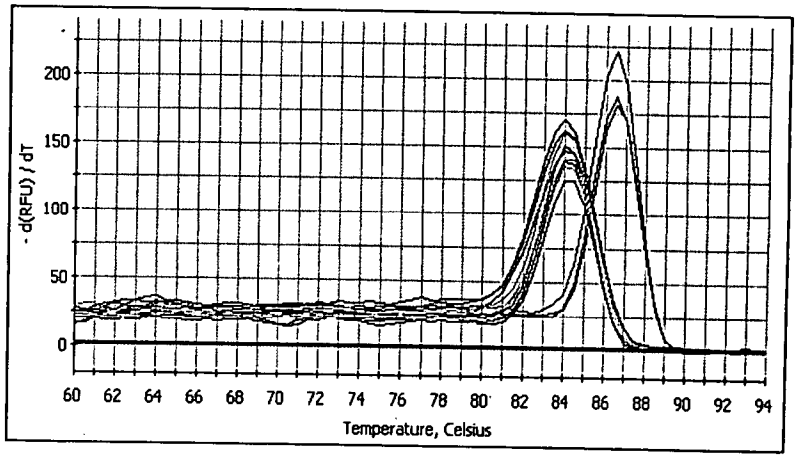
eppendorf

*In touch with life*

Figure 2



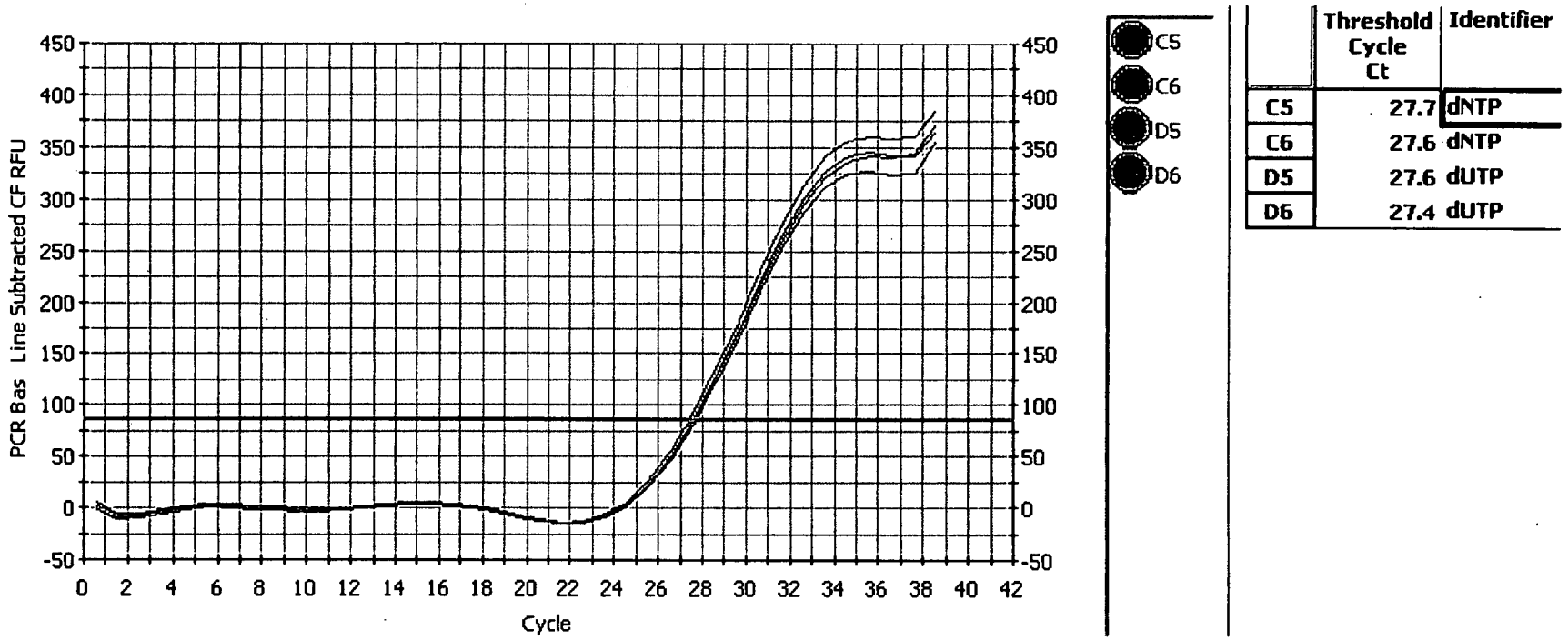




eppendorf

*In touch with life*

Figure 3

# dTTP (10mM) vs dUTP (2.5mM, 7.5mM dTTP)

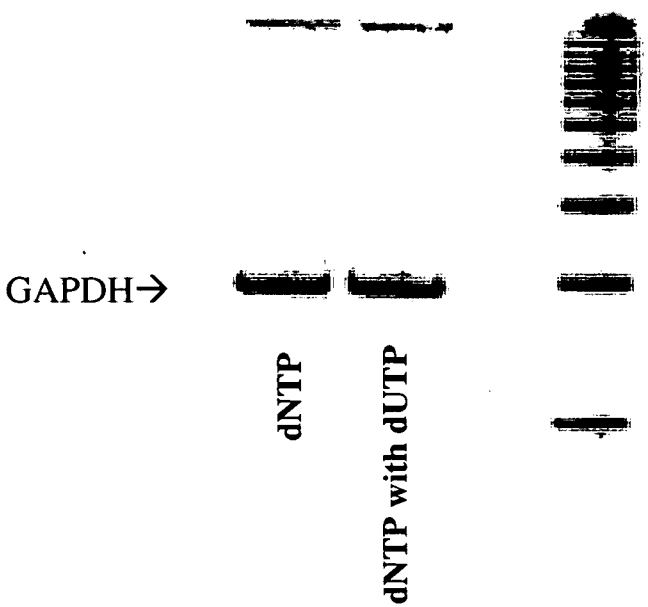



| | Threshold Cycle Ct | Identifier |
|---|---|---|
| C5 | 27.7 | dNTP |
| C6 | 27.6 | dNTP |
| D5 | 27.6 | dUTP |
| D6 | 27.4 | dUTP |

In real-time RT-PCR, the use of dTTP or a combination of dTTP and dUTP does not seem to affect the Ct or RFU significantly.

Figure 4A

# Standard dNTP mix with dTTP (10mM) vs dUTP mix (2.5mM dUTP, 7.5mM dTTP)



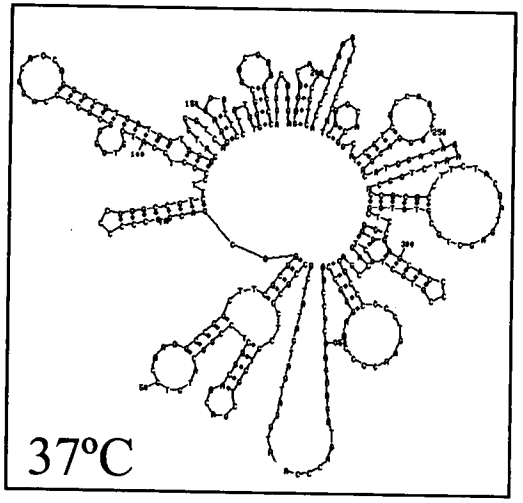

These results show that the addition of dUTP to the dNTP mix does not significantly affect the product yield.

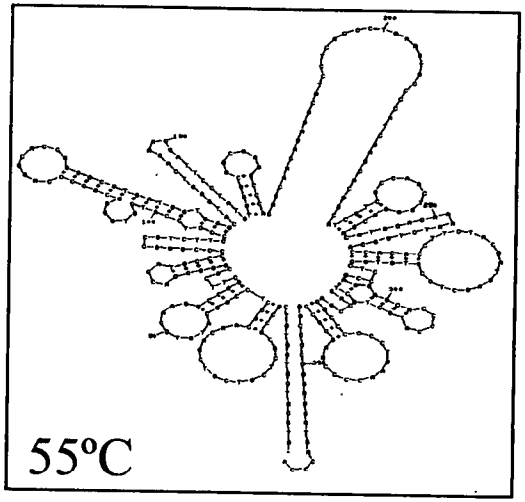
Figure 4B

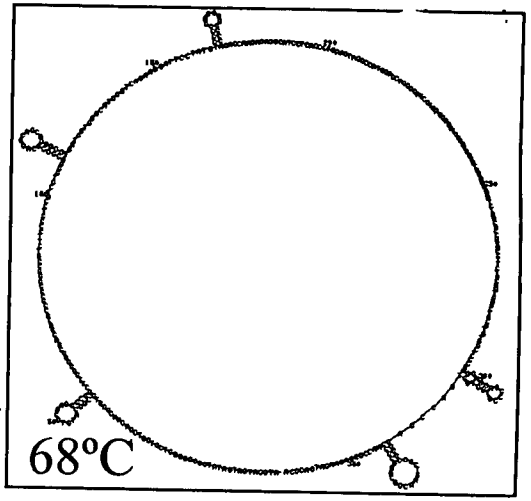
[redacted]

## Beta-Actin mRNA Sequence

atggatgatgatatcgccgcgctcgtcgtcgacaacggctccggcatgtgcaaggccggcttcgcgggcga
cgatgccccccgggccgtcttccctccatcgtggggcgccccaggcaccagggcgtgatggtgggcatg
ggtcagaaggattcctatgtgggcgacgaggcccagagcaagagaggcatcctcaccctgaagtacccca
tcgagcacggcatcgtcaccaactgggacgacatggagaaaatctggcaccacaccttctacaatgagctg
cgtgtggctcccgaggagcaccccgtgctgctgaccgaggccccctgaacccaagggccaaccgcga
gaagatgaccca




eppendorf

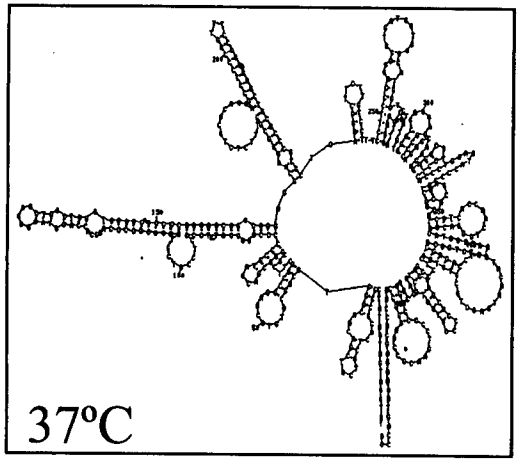
*In touch with life*

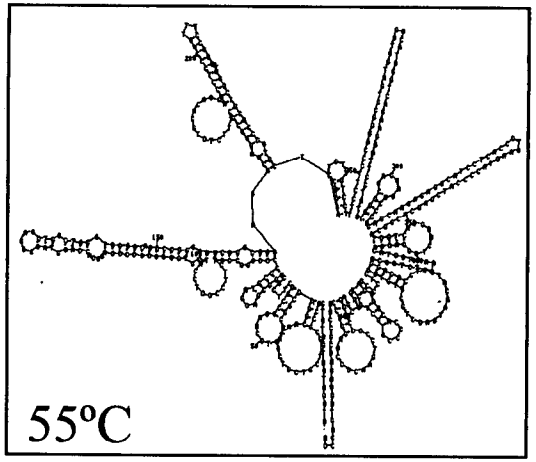
Figure 5A

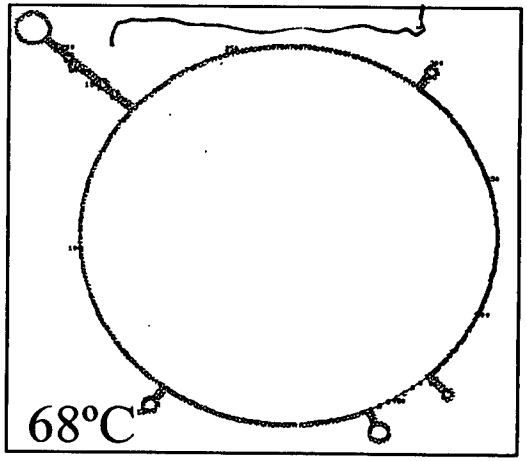
[redacted]

## Beta-Actin partial Gene Sequence w/ Intron

atggatgatgatatcgccgcgctcgtcgtcgacaacggctccggcatgtgcaaggccggcttcgcgggcga
cgatgcccccccgggccgtcttcccctccatcgtggggcgccccaggcaccag|gtaggggagctggctgg
gtggggcagccccgggagcgggcgggaggcaagggcgctttctctgcacaggagcctcccggtttccgg
ggtgggctgcgcccgtgctcagggcttcttgtcctttccttcccag|ggcgtgatggtgggcatgggtcagaa
ggattcctatgtgggcgacgaggcccagagcaagagaggcatcctcaccctgaagtaccccatcgagcac
ggcatcgtcaccaactgggacgacatggagaaaatctggcaccacaccttctacaatgagctgcgtgtggct
cccgaggagcaccccgtgctgctgaccgaggcccccctgaacccaagggccaaccgcgagaagatga
ccca



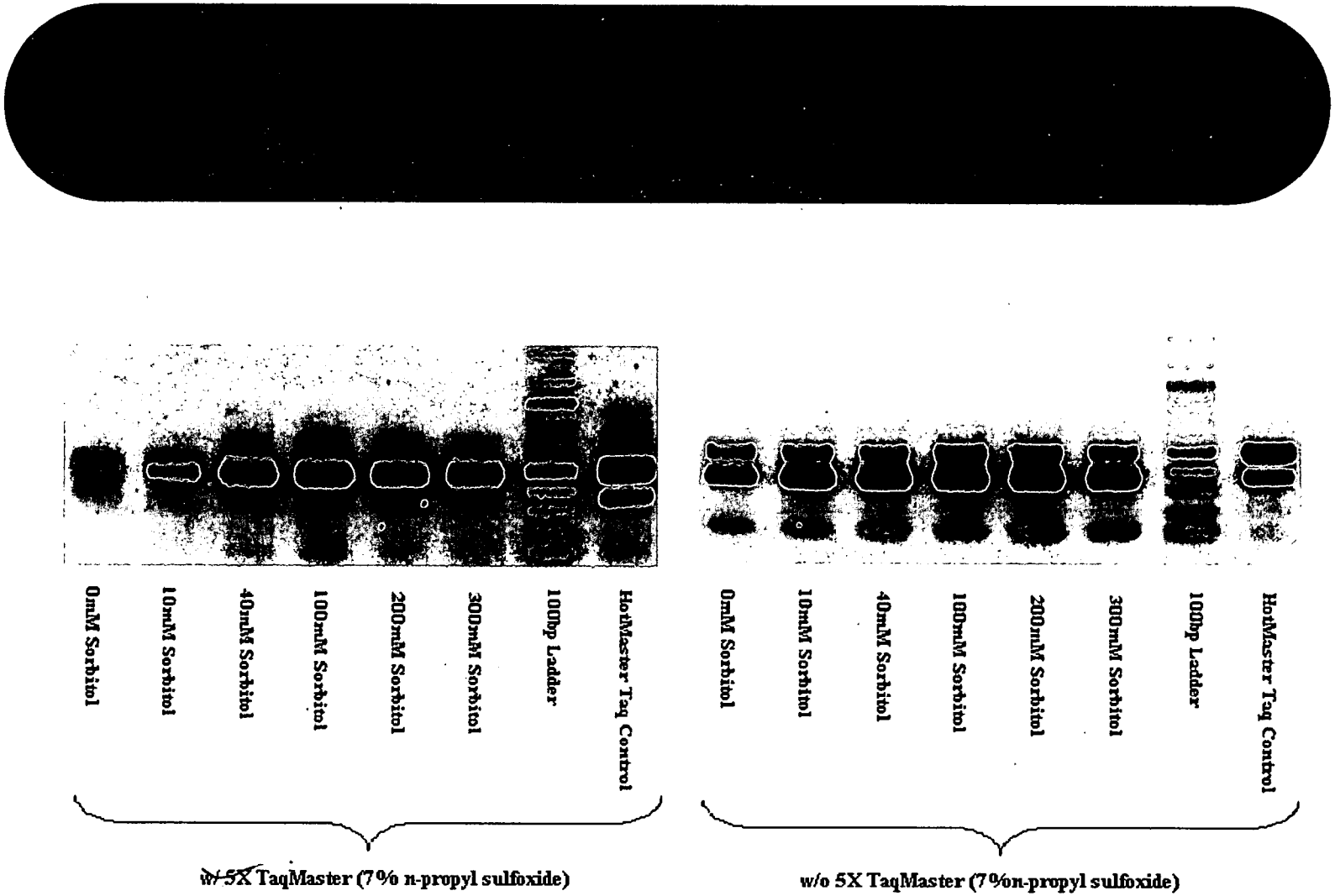




**eppendorf**

***In touch with life***

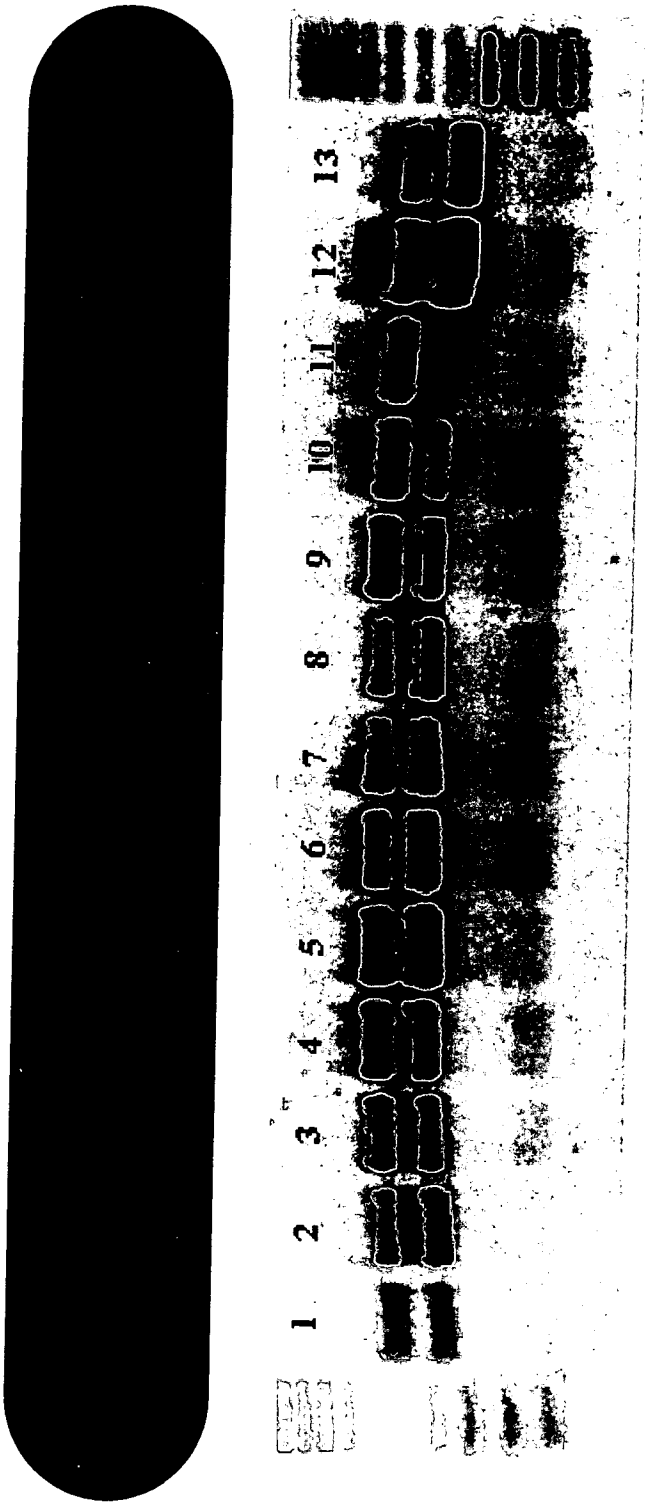
Figure 5B




eppendorf

*In touch with life*

Figure 6




100bp Ladder

25% dUTP HotMaster Control

STND dNTP HotMaster Control

25% dUTP/ 3%DMSO/ 300mM Mann/ SSBP

25% dUTP/ 3%DMSO/ 300mM Sorb/SSBP

25% dUTP/ 300mM Mannitol/ SSBP

25% dUTP/ 300mM Sorbitol/ SSBP

25% dUTP/ 3% DMSO/ SSBP

25% dUTP Mix/ 300mM Mannitol

25% dUTP Mix/ 300mM Sorbitol

25% dUTP Mix/ 3% DMSO

STND dTNP/ 300mM Mannitol

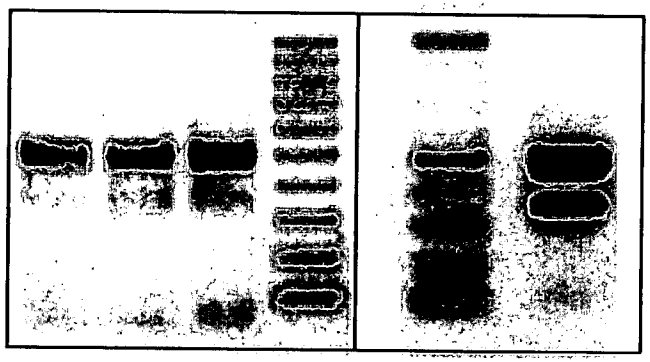
STND dNTP/ 300mM Sorbitol

STND dNTP/ 3% DMSO

100bp Ladder

eppendorf
*In touch with life*

Figure 7A



2% DMSO/ 300mM Sorbitol
2.5% DMSO/ 300mM Sorbitol
3%DMSO/ 300mM Sorbitol
100bp Ladder
100bp Ladder
STND HotMaster Control

| Set Up per 50uL Reaction | | | | |
|---|---|---|---|---|
| Reaction Components | HM Control | 2% DMSO | 2.5% DMSO | 3% DMSO |
| 10X HotMaster Buffer | 5uL | 5uL | 5uL | 5uL |
| HotMaster DNA Taq Polymerase (5U/uL) | .2uL | .2uL | .2uL | .2uL |
| 10mM STND dNTP Mix | 1uL | 1uL | 1uL | 1uL |
| B-Actin Forward Primer (10uM) | 1uL | 1uL | 1uL | 1uL |
| B-Actin Reverse Primer (10uM) | 1uL | 1uL | 1uL | 1uL |
| 1M Sorbitol | 0uL | 15uL | 15uL | 15uL |
| 99.9% DMSO | 0uL | 1uL | 1.25uL | 1.5uL |
| Human Henomic DNA (25ng/uL) | 2uL | 2uL | 2uL | 2uL |
| MBGW | 39.2uL | 23.8uL | 23.55uL | 23.3uL |

**Protocol**

| | | |
|---|---|---|
| 95°C | 60 seconds | |
| 95°C | 20 seconds | 40 cycles |
| 59°C | 20 seconds | |
| 68°C | 20 seconds | |
| 4°C | Hold | |

eppendorf
*In touch with life*

Figure 7B

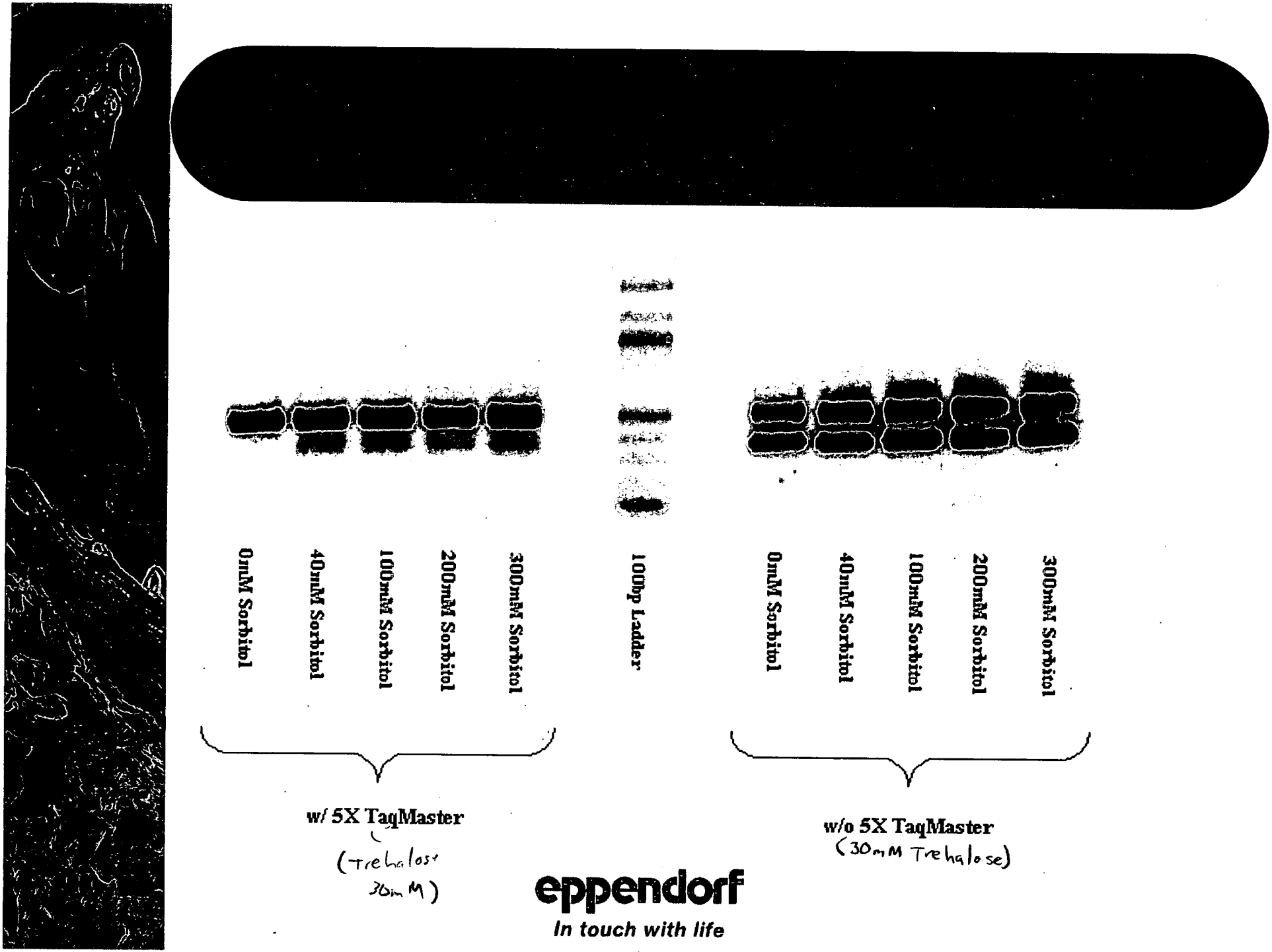
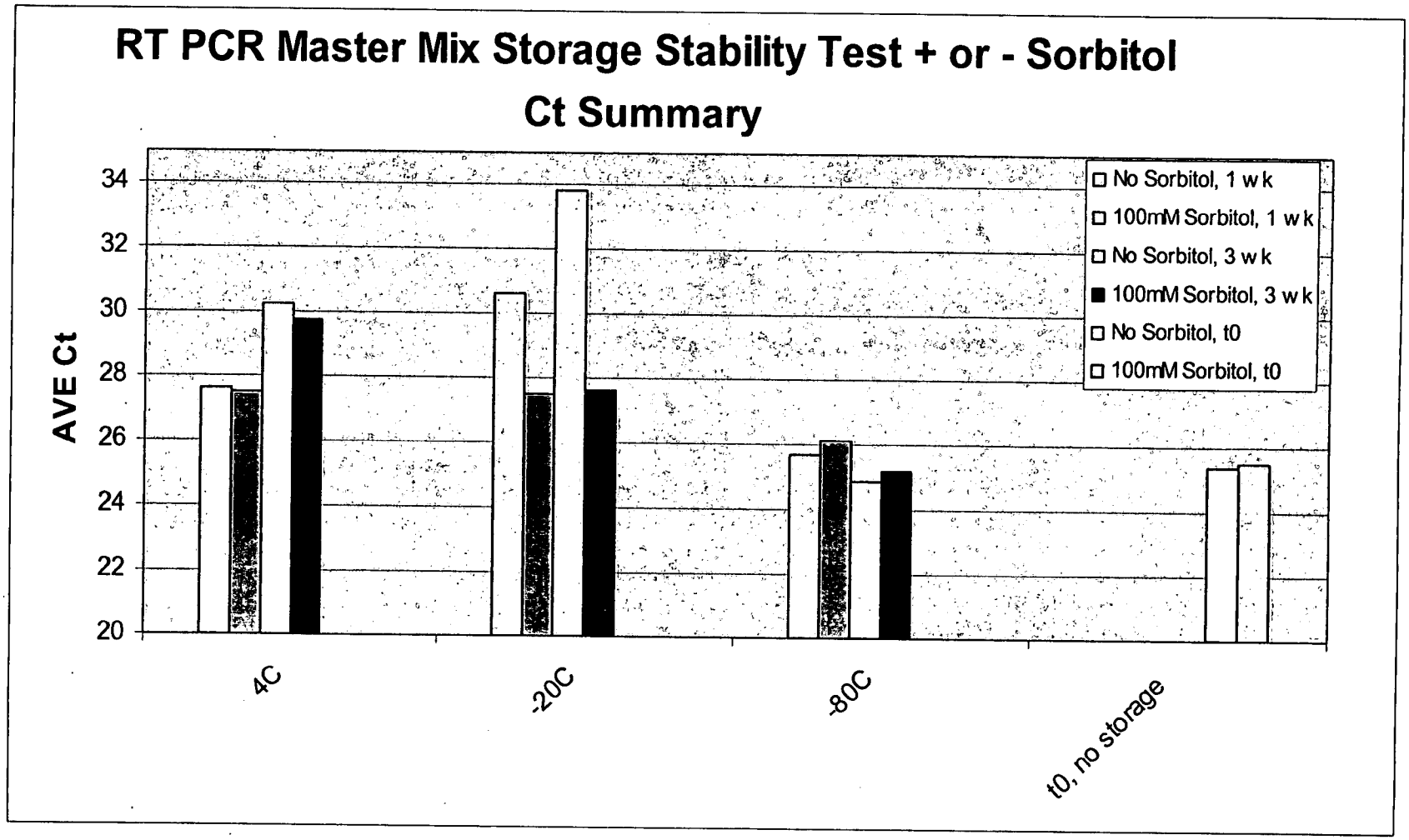
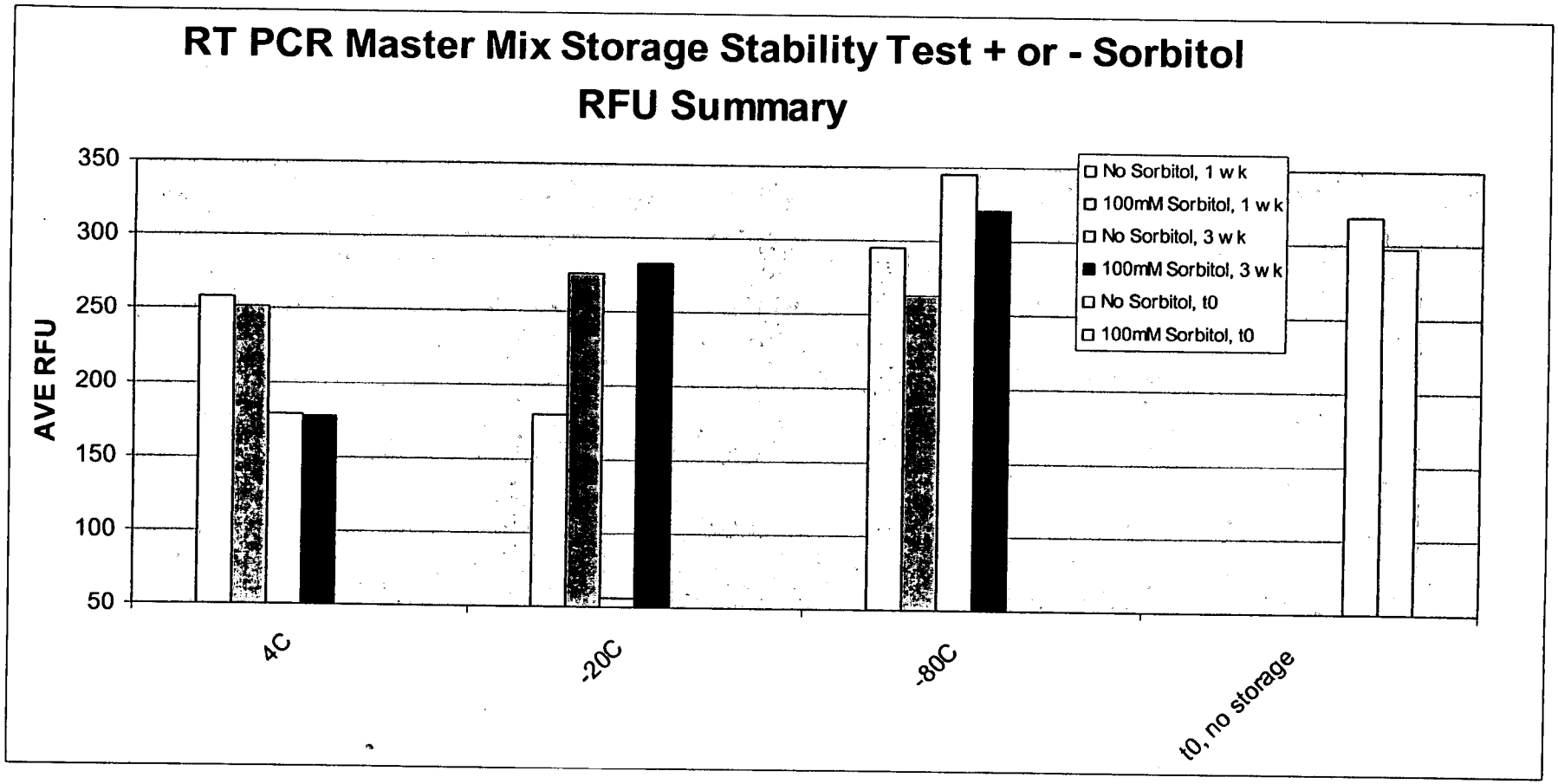

0mM Sorbitol
40mM Sorbitol
100mM Sorbitol
200mM Sorbitol
300mM Sorbitol
100bp Ladder
0mM Sorbitol
40mM Sorbitol
100mM Sorbitol
200mM Sorbitol
300mM Sorbitol
w/ 5X TaqMaster
(Trehalose 30mM)
w/o 5X TaqMaster
(30mM Trehalose)
eppendorf
In touch with life

Figure 7C




| Ct chart | | | | | | |
|---|---|---|---|---|---|---|
| | No Sorbitol, 1 wk | No Sorbitol, 3 wk | 100mM Sorbitol, 1 wk | 100mM Sorbitol, 3 wk | No Sorbitol, t0 | 100mM Sorbitol, t0 |
| 4C | 27.65 | 30.3 | 27.5 | 29.8 | | |
| -20C | 30.65 | 33.8 | 27.5 | 27.6 | | |
| -80C | 25.7 | 24.9 | 26.15 | 25.2 | | |
| t0, no storage | | | | | 25.35 | 25.5 |

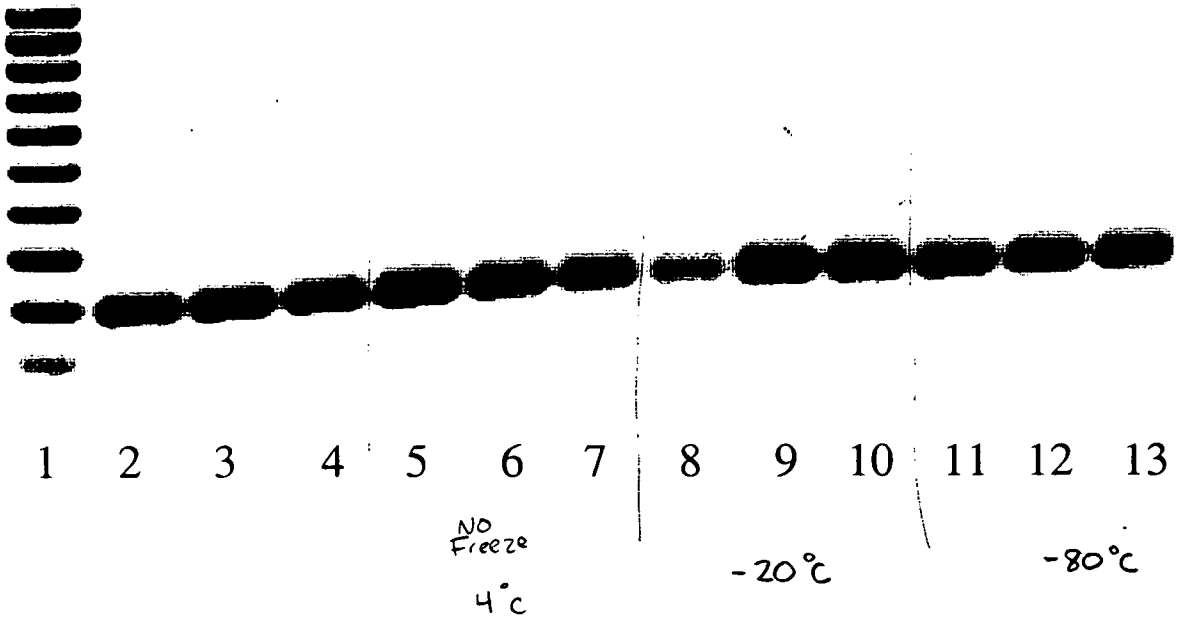
Figure 8A




| RFU chart | | | | | | |
|---|---|---|---|---|---|---|
| | No Sorbitol, 1 wk | No Sorbitol, 3 wk | 100mM Sorbitol, 1 wk | 100mM Sorbitol, 3 wk | No Sorbitol, t0 | 100mM Sorbitol, t0 |
| 4C | 258.02 | 179.5 | 250.63 | 178.4 | | |
| -20C | 180.635 | 56.0 | 275.8 | 282.7 | | |
| -80C | 295.69 | 345.2 | 262.985 | 320.7 | | |
| t0, no storage | | | | | 318.405 | 297.95 |

Figure 8B

# RT PCR Master Mix Storage Stability Test + or - Sorbitol
# t0 vs t = 1 week, Product Yield Comparison




| Lane | Sample |
|---|---|
| 1 | Molecular weight ladder |
| 2 | t0, no sorb |
| 3 | t0, 100mM sorb |
| 4 | t0, 200mM sorb |
| 5 | t=1wk, 4C, no sorb |
| 6 | t=1wk, 4C, 100mM sorb |
| 7 | t=1wk, 4C, 200mM sorb |
| 8 | t=1wk, -20C, no sorb |
| 9 | t=1wk, -20C, 100mM sorb |
| 10 | t=1wk, -20C, 200mM sorb |
| 11 | t=1wk, -80C, no sorb |
| 12 | t=1wk, -80C, 100mM sorb |
| 13 | t=1wk, -80C, 200mM sorb |

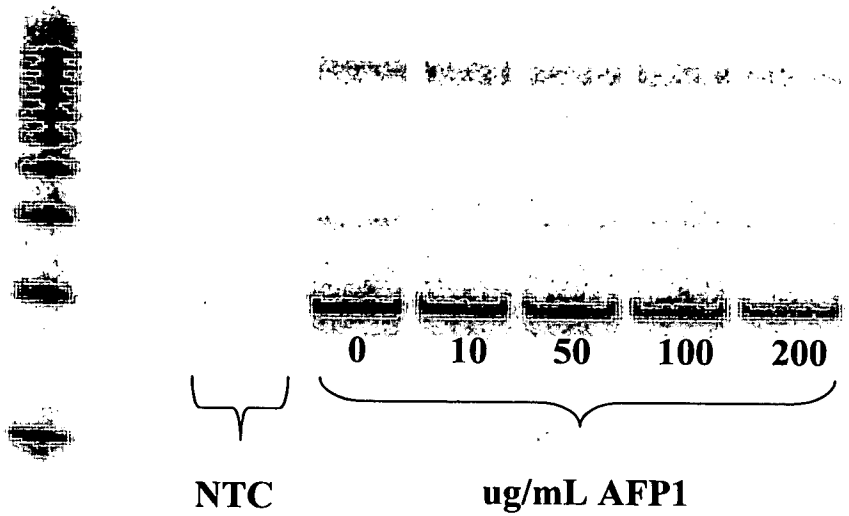
Figure 9A

# RT PCR Master Mix Storage Stability Test + or – Sorbitol
# t = 3 weeks, Product Yield Comparison

| Lane | Sample |
|---|---|
| 1 | Molecular weight ladder |
| 2 | t=3wk, 4C, no sorb |
| 3 | t=3wk, 4C, 100mM sorb |
| 4 | t=3wk, 4C, 200mM sorb |
| 5 | t=3wk, -20C, no sorb |
| 6 | t=3wk, -20C, 100mM sorb |
| 7 | t=3wk, -20C, 200mM sorb |
| 8 | t=3wk, -80C, no sorb |
| 9 | t=3wk, -80C, 100mM sorb |
| 10 | t=3wk, -80C, 200mM sorb |

Figure 9B

# Real-Time PCR Titration with AFP1




50µg/mL AFP1 is the highest concentration that does not affect product yield.

Figure 10

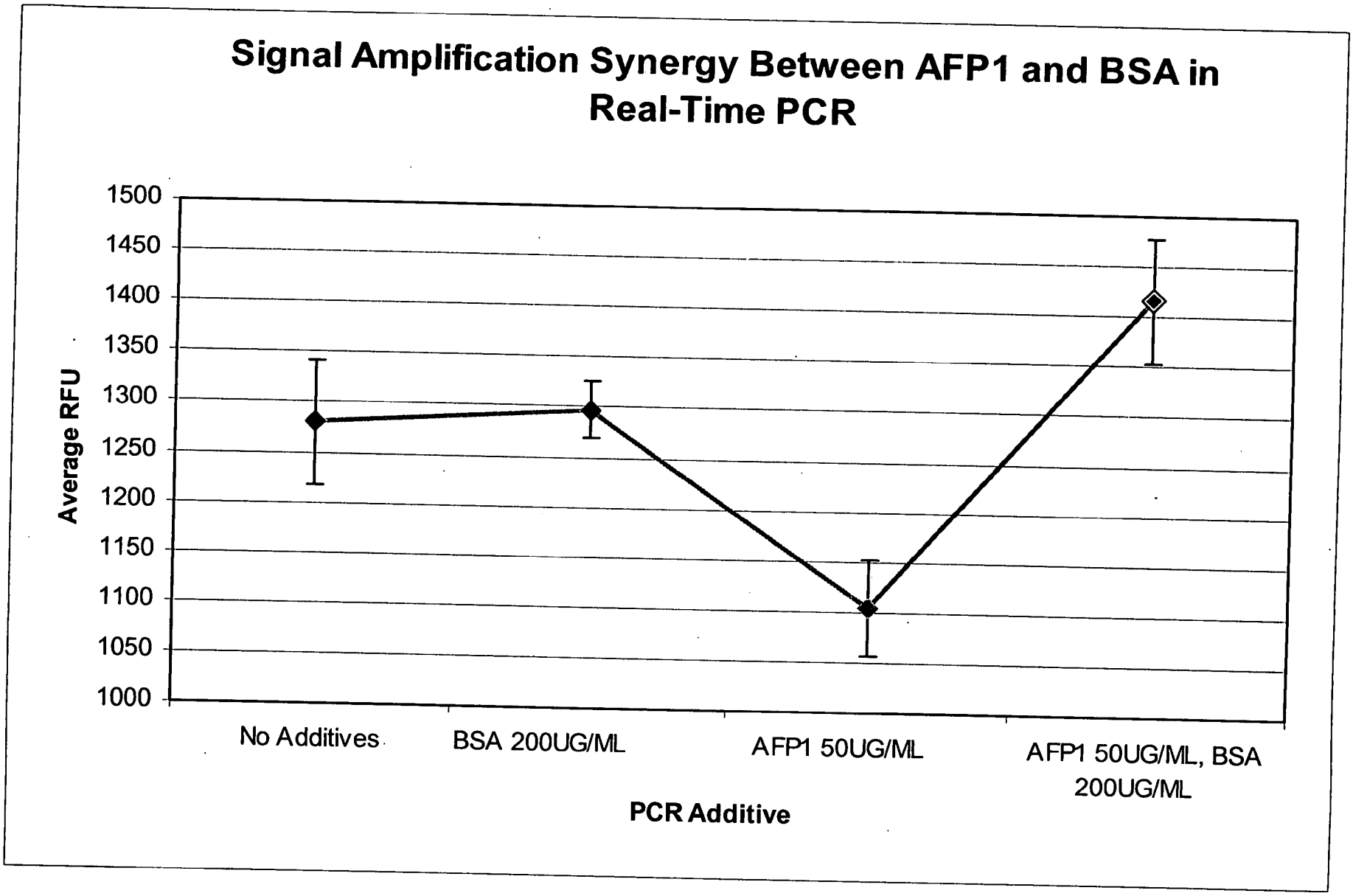

Signal Amplification Synergy Between AFP1 and BSA in Real-Time PCR
Average RFU
1500
1450
1400
1350
1300
1250
1200
1150
1100
1050
1000
No Additives
BSA 200UG/ML
AFP1 50UG/ML
AFP1 50UG/ML, BSA 200UG/ML
PCR Additive

Figure 11A

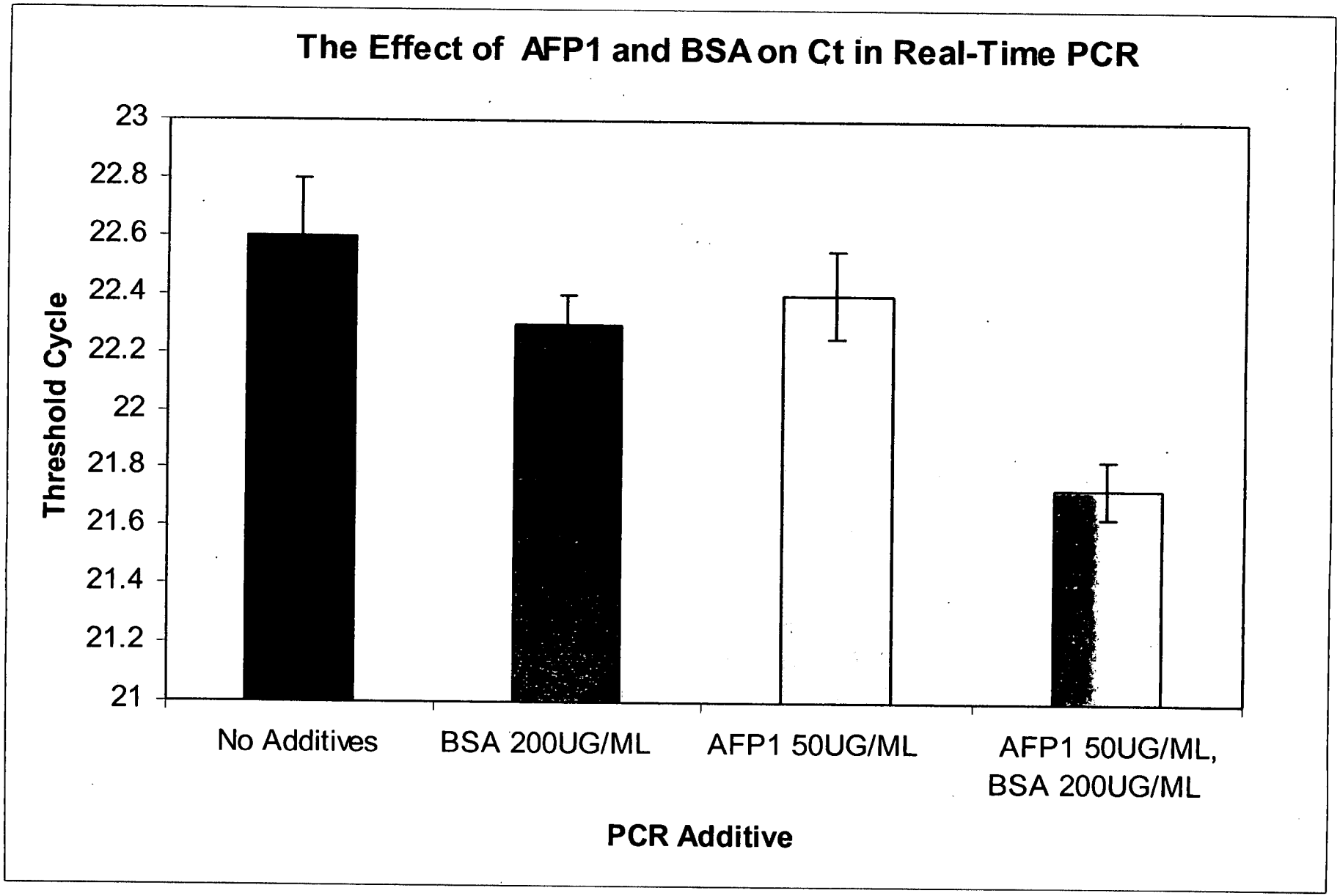

The Effect of AFP1 and BSA on Ct in Real-Time PCR
Threshold Cycle
23
22.8
22.6
22.4
22.2
22
21.8
21.6
21.4
21.2
21
No Additives
BSA 200UG/ML
AFP1 50UG/ML
AFP1 50UG/ML, BSA 200UG/ML
PCR Additive

Figure 11 B

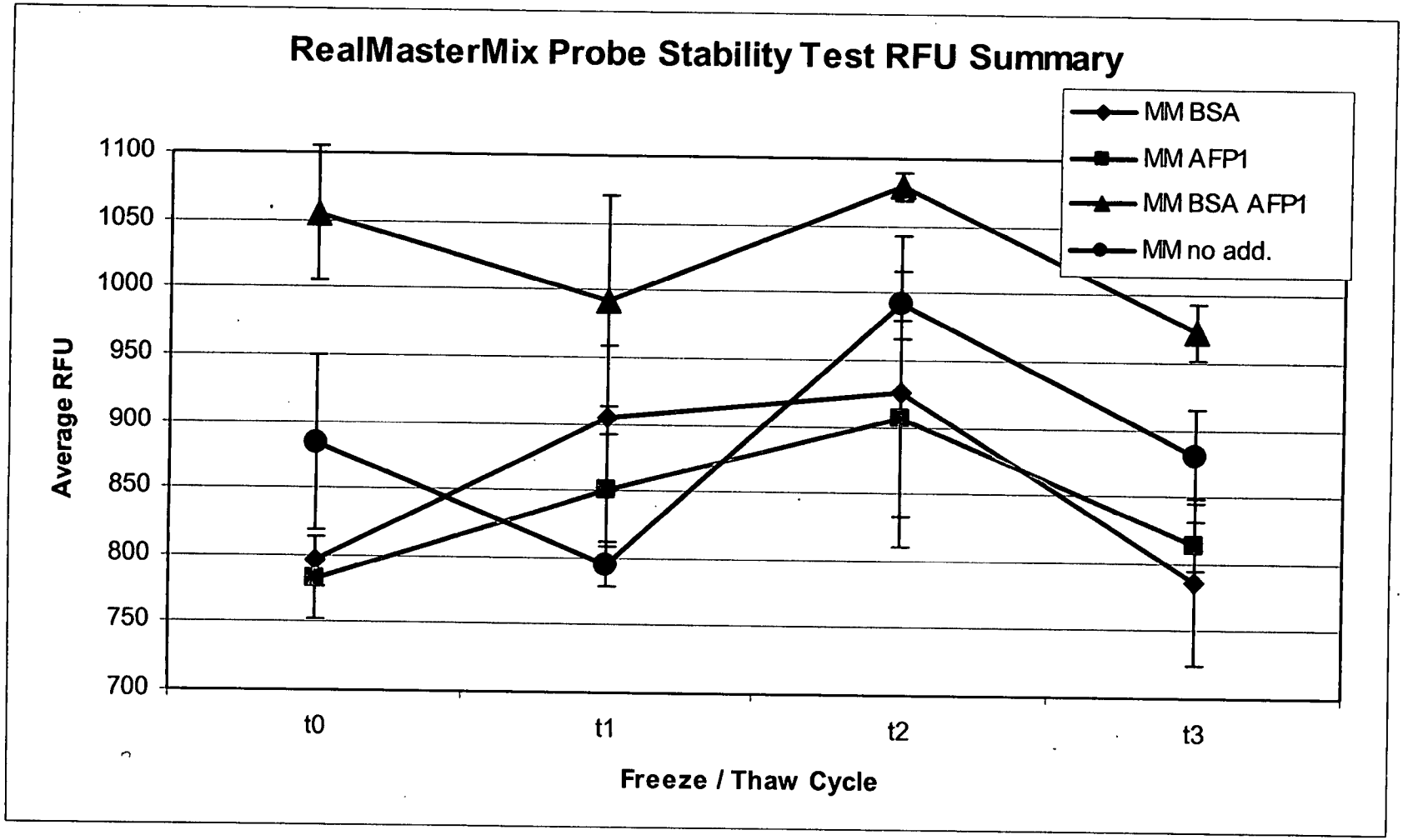

RealMasterMix Probe Stability Test RFU Summary
MM BSA
MM AFP1
MM BSA AFP1
MM no add.
1100
1050
1000
950
900
850
800
750
700
Average RFU
t0
t1
t2
t3
Freeze / Thaw Cycle

Figure 12A

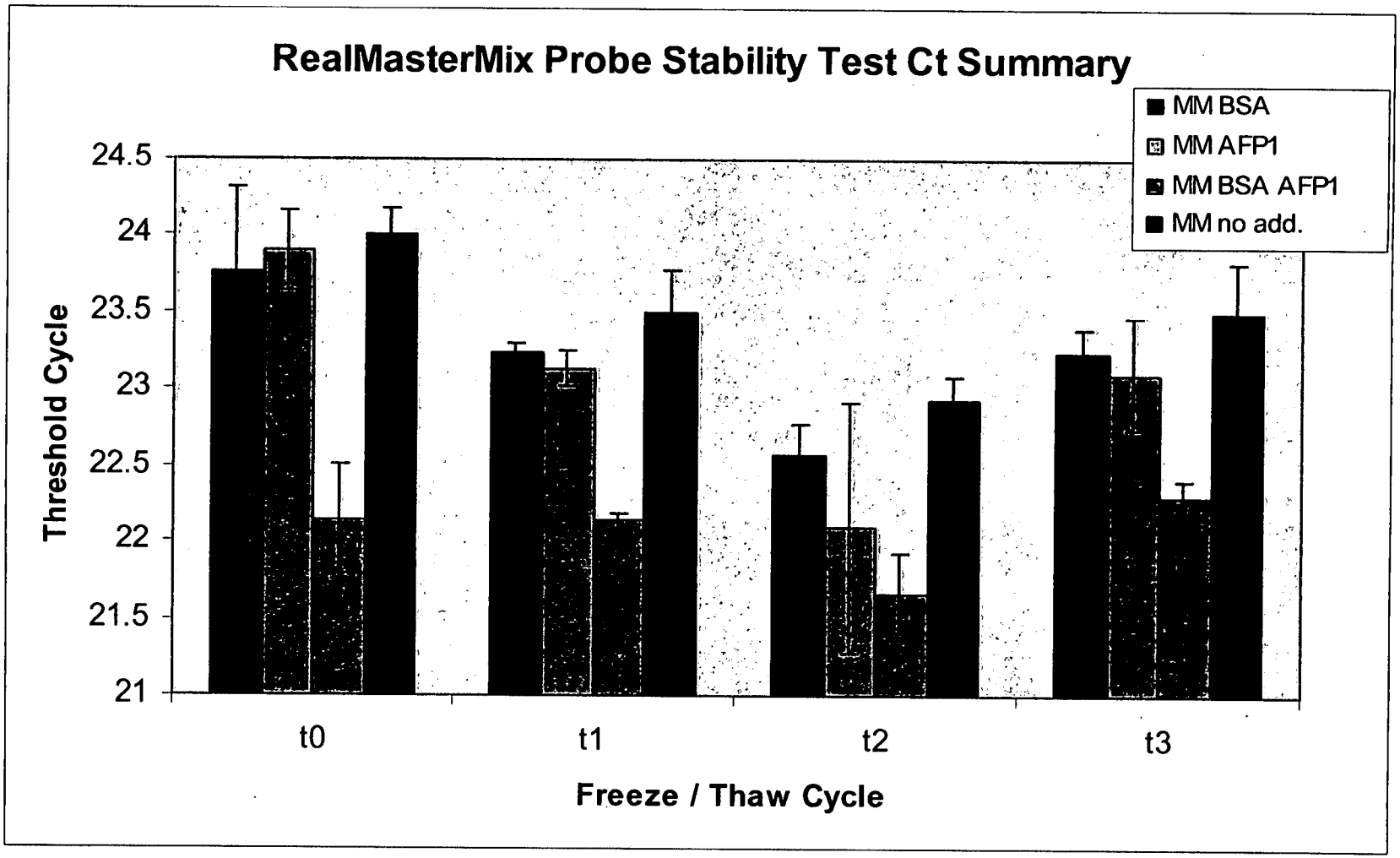

RealMasterMix Probe Stability Test Ct Summary
MM BSA
MM AFP1
MM BSA AFP1
MM no add.
Threshold Cycle
24.5
24
23.5
23
22.5
22
21.5
21
t0
t1
t2
t3
Freeze / Thaw Cycle

Figure 12B

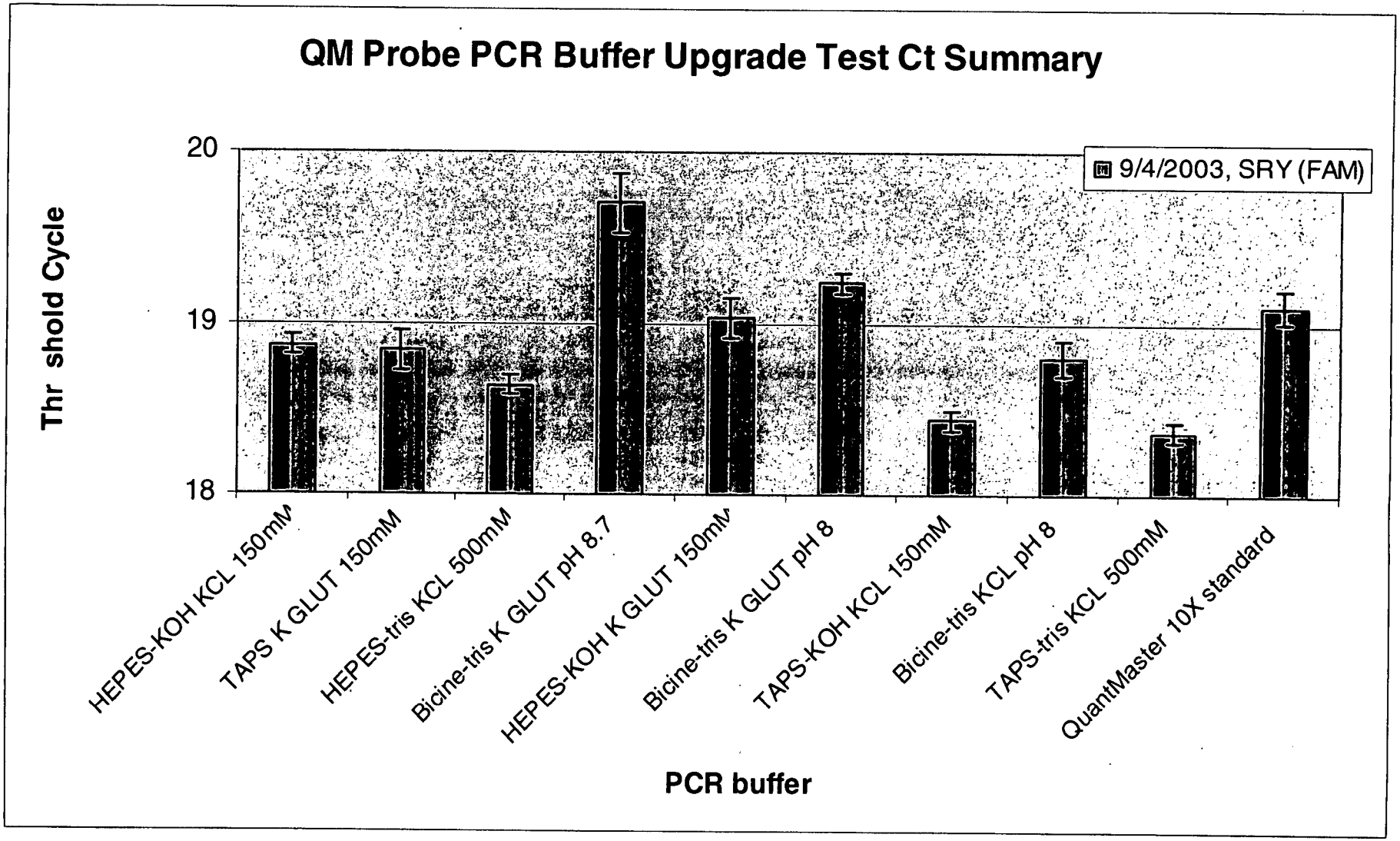

QM Probe PCR Buffer Upgrade Test Ct Summary
Thr shold Cycle
20
19
18
9/4/2003, SRY (FAM)
HEPES-KOH KCL 150mM
TAPS K GLUT 150mM
HEPES-tris KCL 500mM
Bicine-tris K GLUT pH 8.7
HEPES-KOH K GLUT 150mM
Bicine-tris K GLUT pH 8
TAPS-KOH KCL 150mM
Bicine-tris KCL pH 8
TAPS-tris KCL 500mM
QuantMaster 10X standard
PCR buffer

Figure 13A

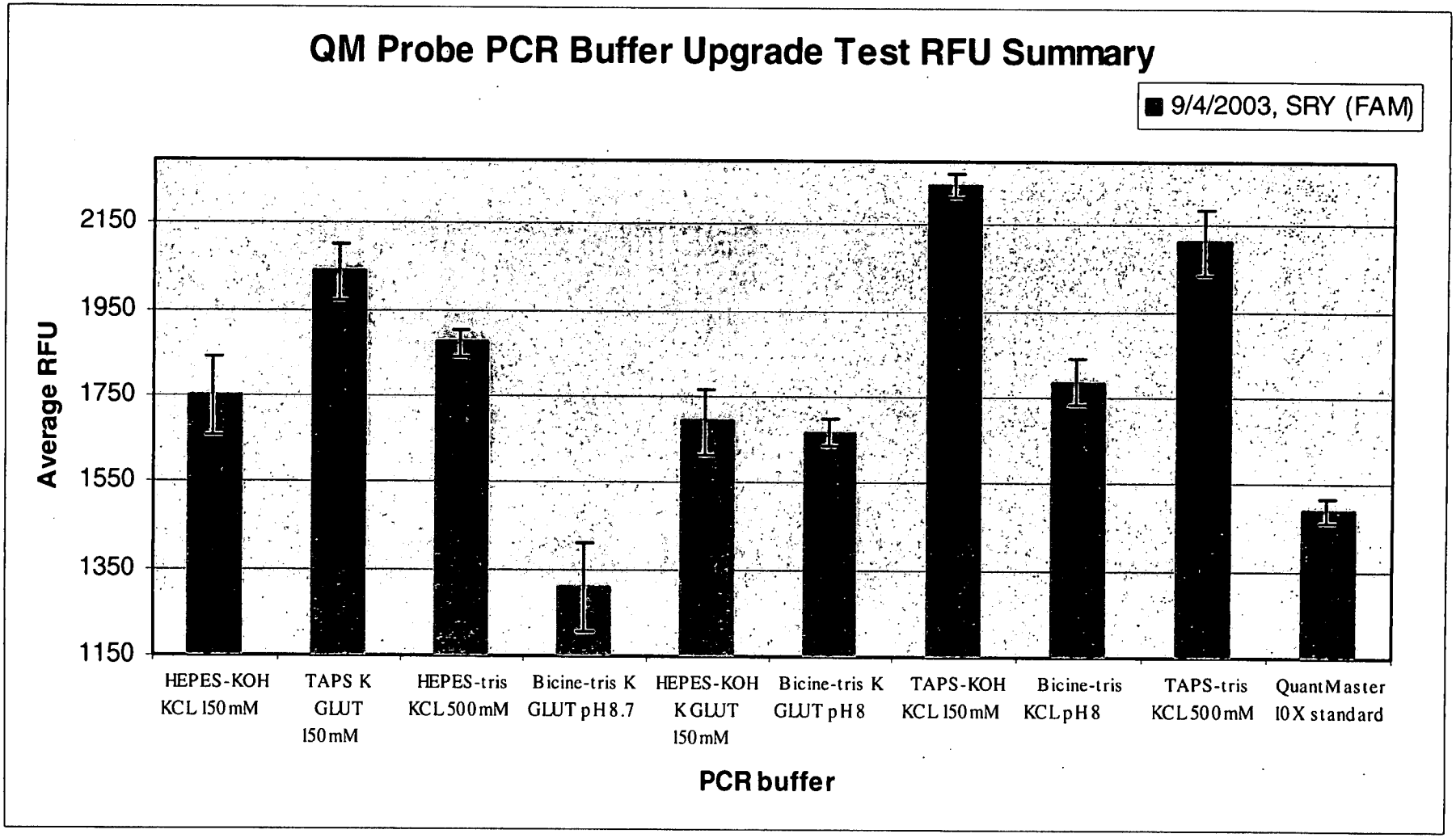

QM Probe PCR Buffer Upgrade Test RFU Summary
9/4/2003, SRY (FAM)
Average RFU
2150
1950
1750
1550
1350
1150
HEPES-KOH KCL 150 mM
TAPS K GLUT 150 mM
HEPES-tris KCL 500 mM
Bicine-tris K GLUT pH 8.7
HEPES-KOH K GLUT 150 mM
Bicine-tris K GLUT pH 8
TAPS-KOH KCL 150 mM
Bicine-tris KCL pH 8
TAPS-tris KCL 500 mM
QuantMaster 10X standard
PCR buffer

Figure 13B

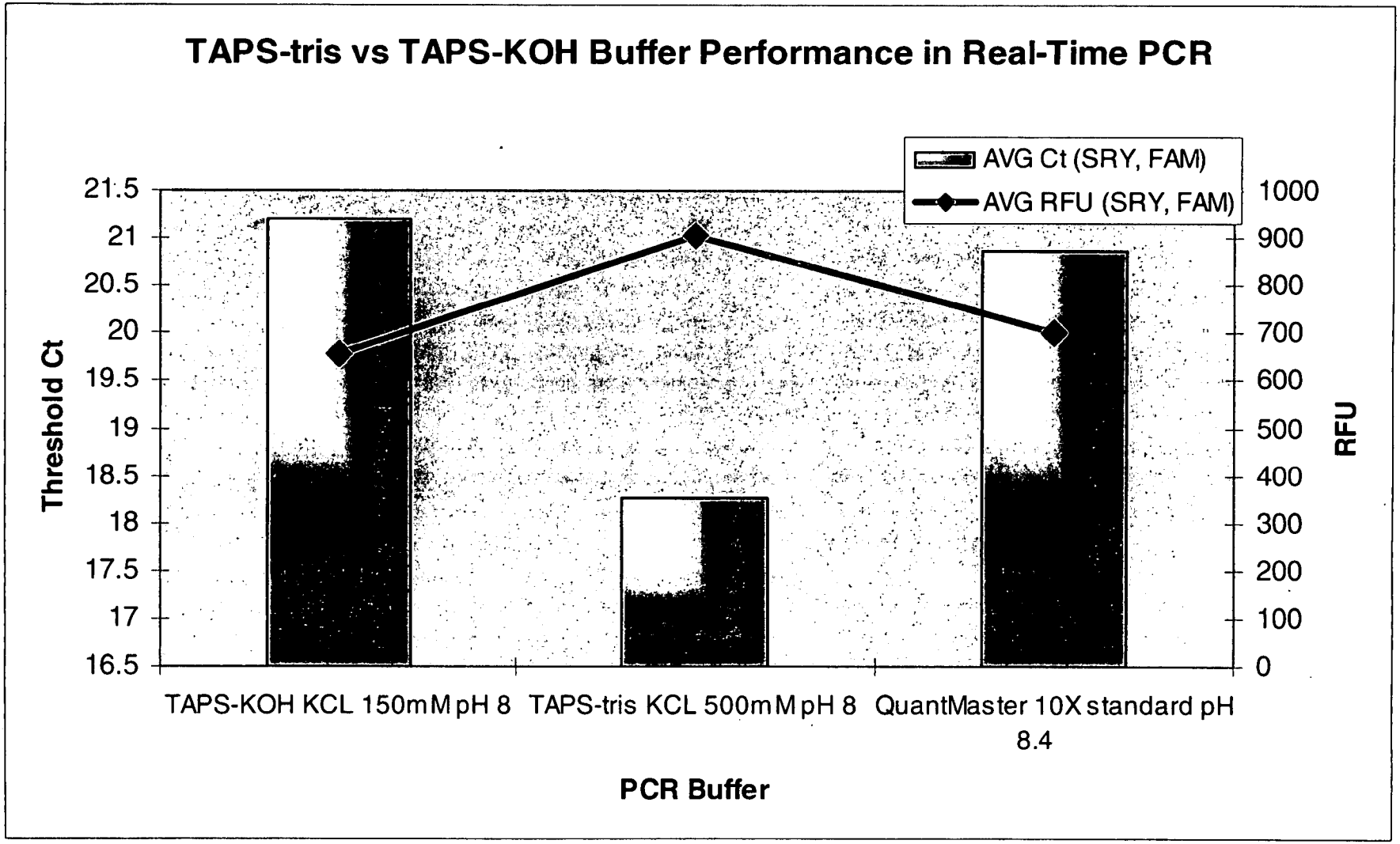

TAPS-tris vs TAPS-KOH Buffer Performance in Real-Time PCR
AVG Ct (SRY, FAM)
AVG RFU (SRY, FAM)
Threshold Ct
RFU
21.5
21
20.5
20
19.5
19
18.5
18
17.5
17
16.5
1000
900
800
700
600
500
400
300
200
100
0
TAPS-KOH KCL 150mM pH 8
TAPS-tris KCL 500mM pH 8
QuantMaster 10X standard pH 8.4
PCR Buffer

Figure 14

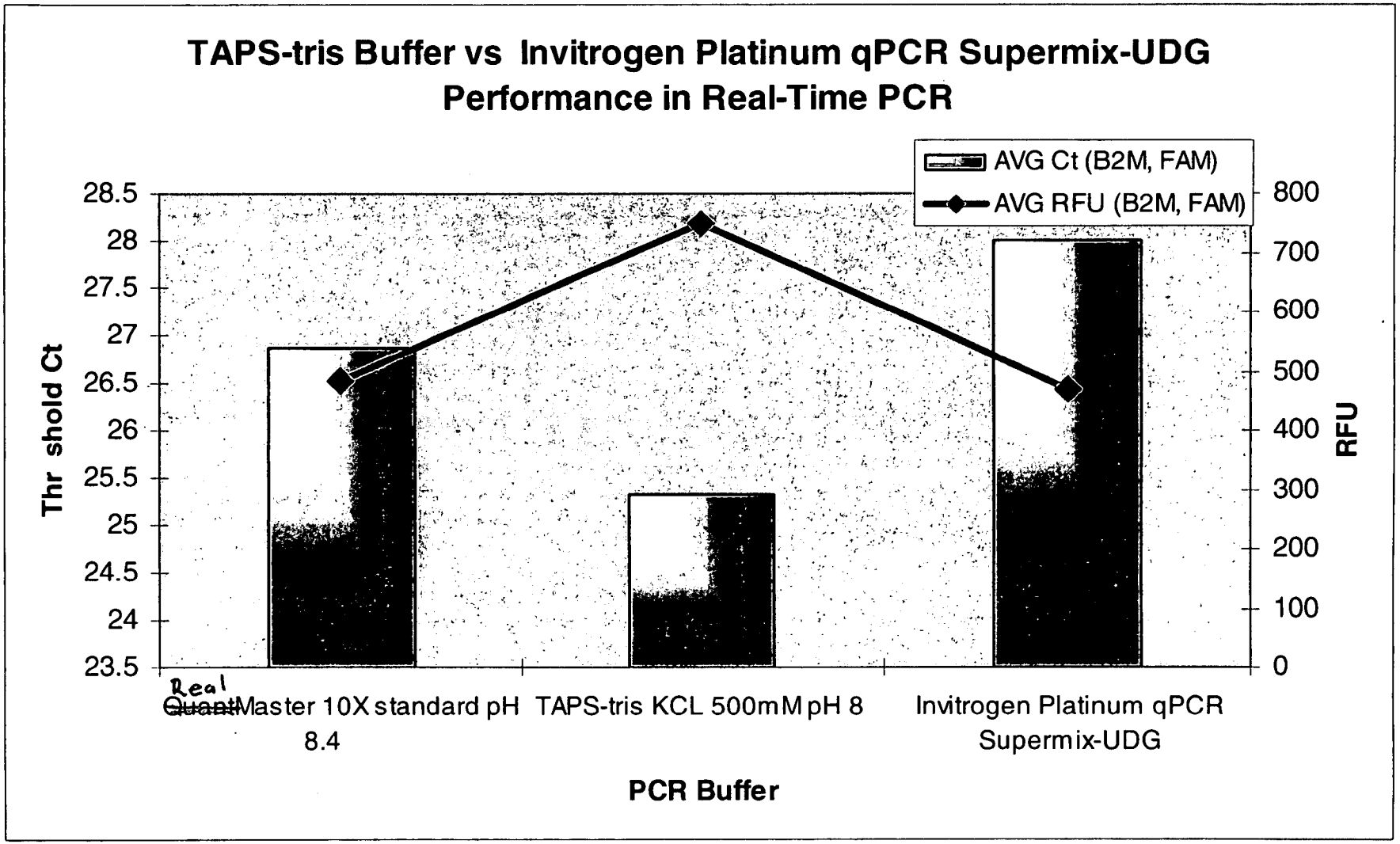

TAPS-tris Buffer vs Invitrogen Platinum qPCR Supermix-UDG Performance in Real-Time PCR
AVG Ct (B2M, FAM)
AVG RFU (B2M, FAM)
Thr shold Ct
RFU
28.5
28
27.5
27
26.5
26
25.5
25
24.5
24
23.5
800
700
600
500
400
300
200
100
0
Real
QuantMaster 10X standard pH 8.4
TAPS-tris KCL 500mM pH 8
Invitrogen Platinum qPCR Supermix-UDG
PCR Buffer

Figure 15

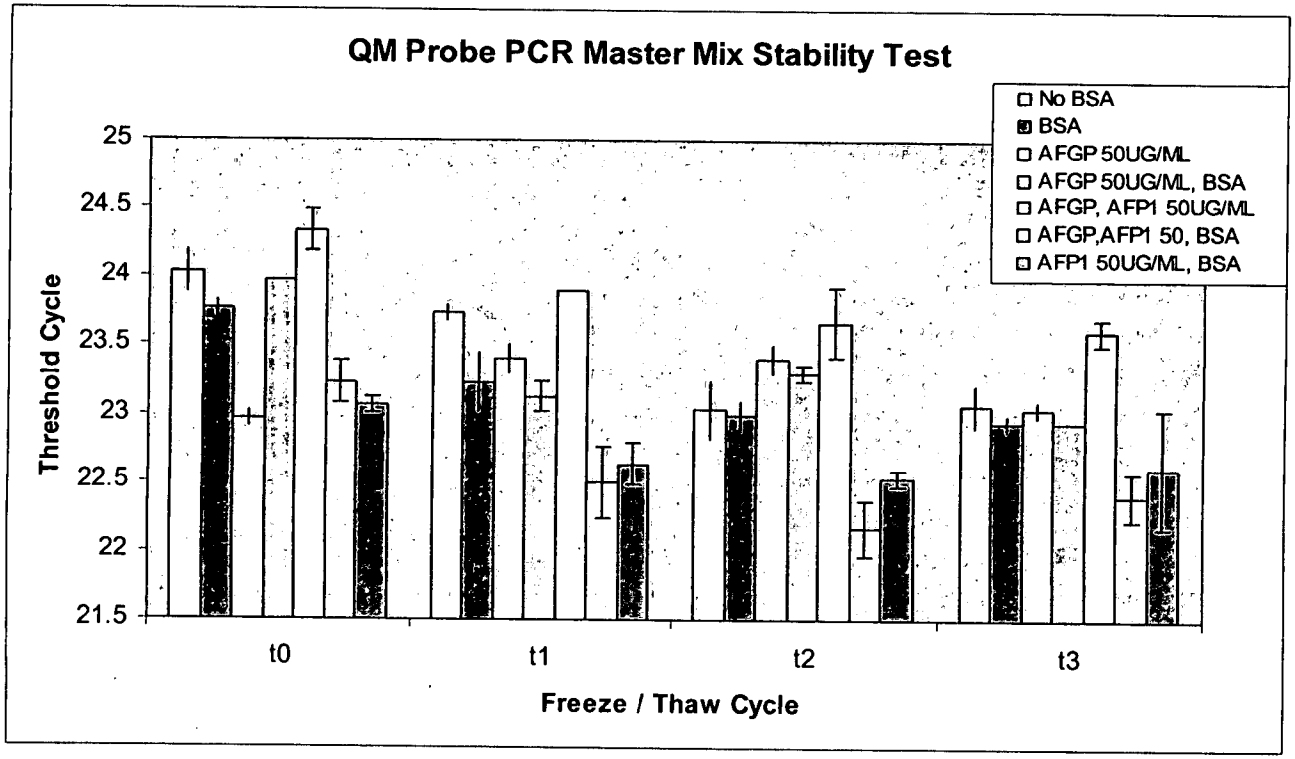
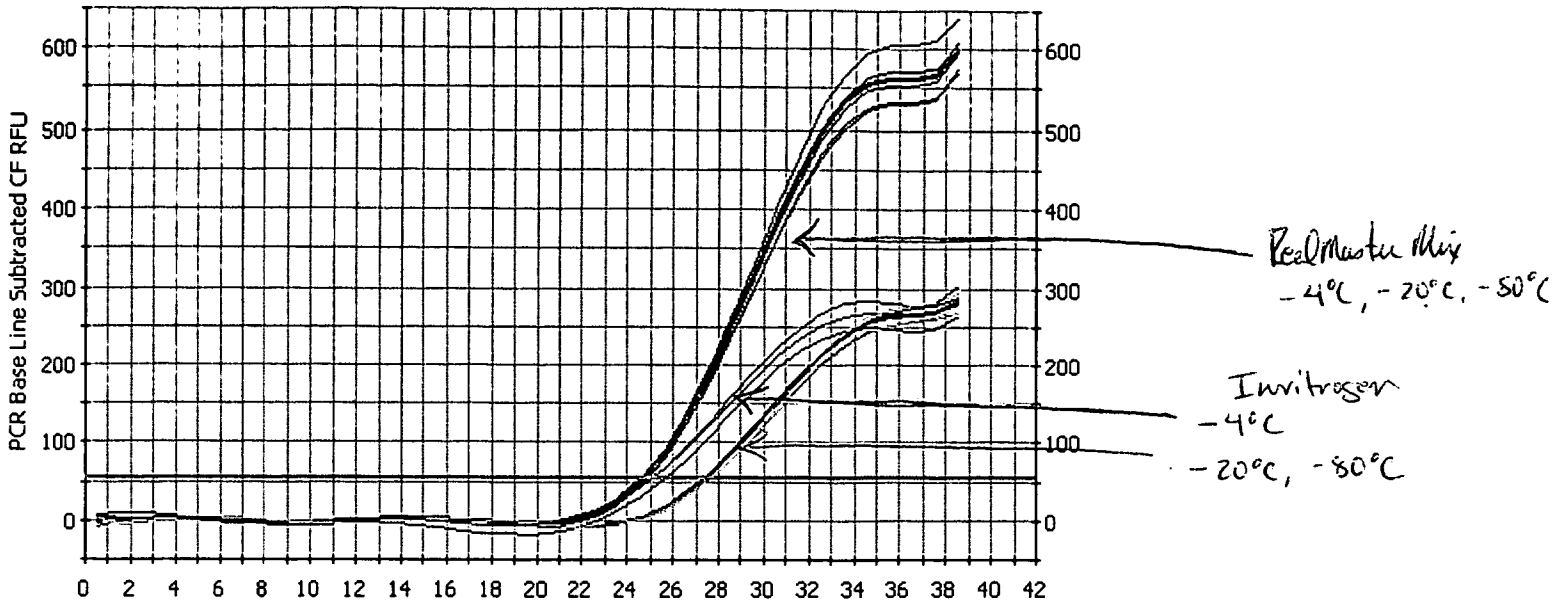

QM Probe PCR Master Mix Stability Test
No BSA
BSA
AFGP 50UG/ML
AFGP 50UG/ML, BSA
AFGP, AFP1 50UG/ML
AFGP,AFP1 50, BSA
AFP1 50UG/ML, BSA
Threshold Cycle
25
24.5
24
23.5
23
22.5
22
21.5
t0
t1
t2
t3
Freeze / Thaw Cycle

Figure 16




6 Week Long term Stability Study

Figure 17

# APPLICATION DATA SHEET

## Application Information

| | |
|---|---|
| Application Type:: | Provisional |
| Subject Matter:: | Utility |
| CD-ROM or CD-R?:: | None |
| Title:: | METHODS AND COMPOSITIONS TO ENHANCE AMPLIFICATION EFFICIENCY AND SIGNAL |
| Attorney Docket No.:: | 34902/US |
| Request for Early Publication?:: | No |
| Request for Non-Publication?:: | No |
| Suggested Drawing Figure:: | |
| Total Drawing Sheets:: | 26 |
| Small Entity:: | No |
| Petition included?:: | No |
| Secrecy Order in Parent Appl.?:: | No |

## Applicant Information

| | |
|---|---|
| Applicant Authority Type:: | Inventor |
| Primary Citizenship Country:: | USA |
| Status:: | Full Capacity |
| Given Name :: | Ryan Smith |
| Family Name :: | Westberry |
| City of Residence:: | Westminster |
| Country of Residence:: | Colorado, USA |
| Street of Mailing Address:: | 10730 Eliot Circle, #203 |
| City of Mailing Address:: | Westminster |
| State of Mailing Address:: | Colorado |
| Country of Mailing Address:: | USA |
| Citizenship Country:: | USA |

| | |
|---|---|
| Applicant Authority Type:: | Inventor |
| Primary Citizenship Country:: | USA |
| Status:: | Full Capacity |
| Given Name :: | Lars-Erik |
| Family Name :: | Peters |
| City of Residence:: | Lafayette |
| Country of Residence:: | Colorado, USA |
| Street of Mailing Address:: | 210 East Geneseo |
| City of Mailing Address:: | Lafayette |
| State of Mailing Address:: | Colorado |
| Country of Mailing Address:: | USA |
| Citizenship Country:: | Germany |

| | |
|---|---|
| Applicant Authority Type:: | Inventor |
| Primary Citizenship Country:: | USA |
| Status:: | Full Capacity |
| Given Name :: | Jessica Jaclyn |
| Family Name :: | Goodrich |
| City of Residence:: | Lafayette |
| Country of Residence:: | Colorado, USA |
| Street of Mailing Address:: | 707 Julian Circle |
| City of Mailing Address:: | Lafayette |
| State of Mailing Address:: | Colorado |
| Country of Mailing Address:: | USA |
| Citizenship Country:: | USA |

**Correspondence Information**

Correspondence Customer No.:: 20686

Phone Number:: (303) 629-3400

Fax Number:: (303) 629-3450

**Representative Information**

| | |
|---|---|
| Representative Customer Number:: | 20686 |

**Foreign Priority Information**

| Country:: | Application Number:: | Filing Date:: | Priority Claimed:: |
|---|---|---|---|
| | | | |

**Domestic Priority Information**

| Application:: | Continuity Type:: | Parent Application:: | Parent Filing Date:: |
|---|---|---|---|
| This Application is a | | | |

**Assignee Information**

Assignee Name::

Street of Address::

City of Mailing Address::

State of Mailing Address::

Country of Mailing Address::